EXHIBIT D

US006759245B1

(12) **United States Patent**
Toner et al.

(10) **Patent No.: US 6,759,245 B1**
(45) **Date of Patent: Jul. 6, 2004**

(54) **CELL CULTURE SYSTEMS AND METHODS FOR ORGAN ASSIST DEVICES**

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(73) Assignees: **The General Hospital Corporation**, Boston, MA (US); **Organogenesis Inc.**, Canton, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/599,891**

(22) Filed: **Jun. 21, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/140,125, filed on Jun. 21, 1999, provisional application No. 60/140,239, filed on Jun. 21, 1999, and provisional application No. 60/181,634, filed on Feb. 10, 2000.

(51) **Int. Cl.**[7] **C12N 5/00**

(52) **U.S. Cl.** **435/401**; 435/284.1; 435/297.1; 435/297.2; 435/402; 435/395; 435/399

(58) **Field of Search** 435/401, 402, 435/395, 399, 284.1, 297.1, 297.2

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Primary Examiner—Leon B. Lankford, Jr.
(74) *Attorney, Agent, or Firm*—Fish & Richardson P.C.

(57) **ABSTRACT**

The invention features modular cell culturing devices including one or more flat-plate modules, and is based on the discovery that if the flows of liquid medium and oxygenated fluid are separated by a gas-permeable, liquid-impermeable membrane, and the cells are grown attached to the liquid side of the membrane, the device can be used to culture cells with transport of oxygen through the membrane (i.e., direct oxygenation), without regard for the flow rate of the liquid medium passing through the device. The new flow-through cell culturing devices can thus be used to culture cells, e.g., hepatocytes, with high levels of cell function in organ, e.g., liver, assist systems, for production of cells, for production of cell-derived products, such as, proteins or viruses, or for systems to treat biological liquids to remove toxins, such as, ammonia, or add cell-synthesized products, or both.

18 Claims, 26 Drawing Sheets

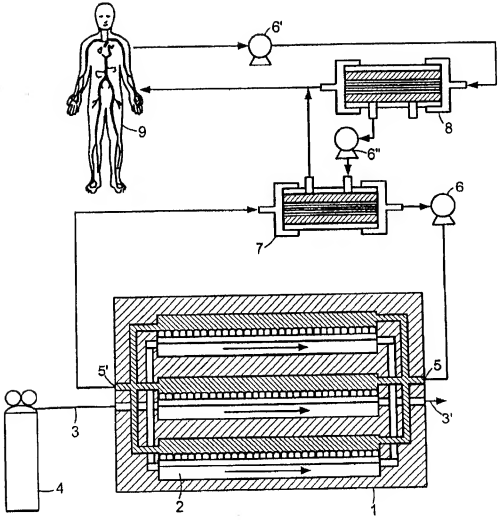


FIG. 1

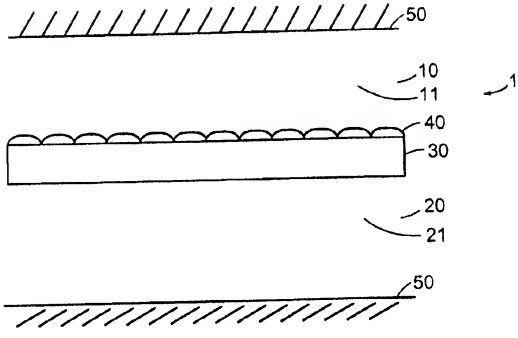


FIG. 2

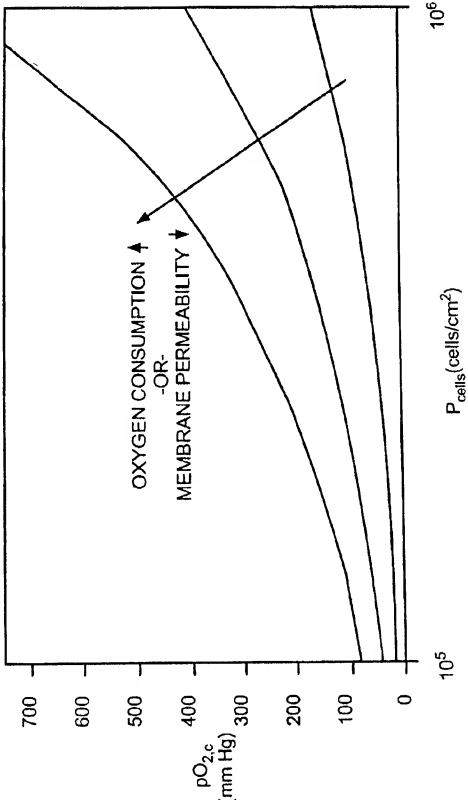

700
600
500
400
300
200
100
0
$pO_{2,c}$ (mm Hg)
OXYGEN CONSUMPTION
-OR-
MEMBRANE PERMEABILITY
10^5
10^6
P_{cells} (cells/cm^2)

FIG. 3

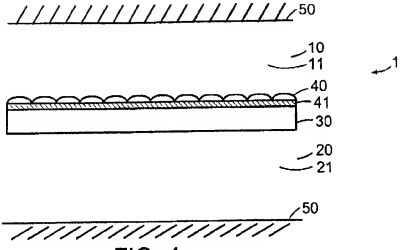


FIG. 4a

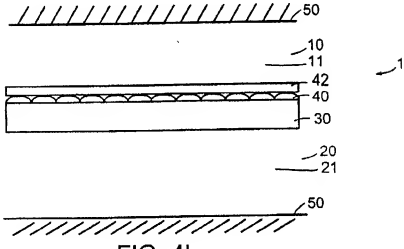


FIG. 4b

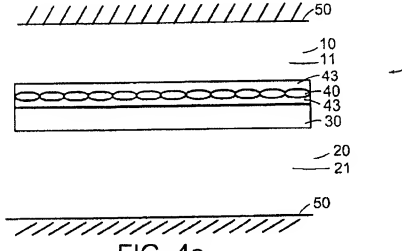


FIG. 4c

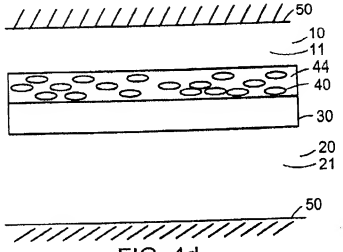


FIG. 4d

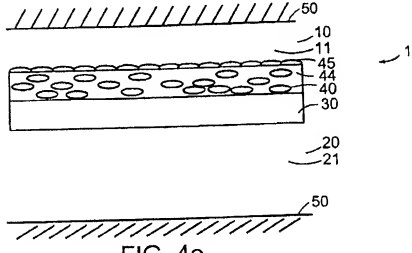


FIG. 4e

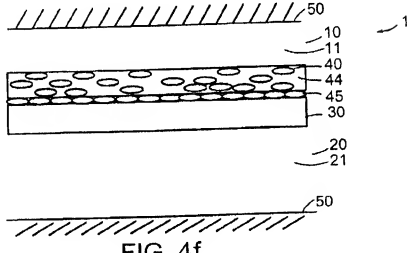


FIG. 4f

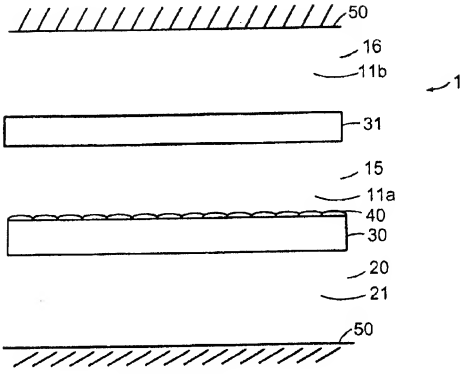


FIG. 5a

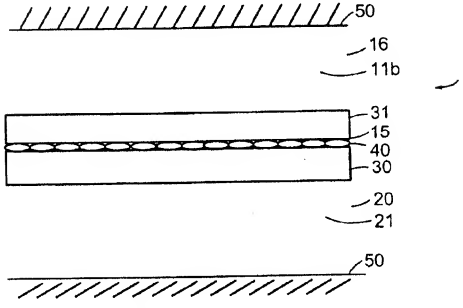


FIG. 5b

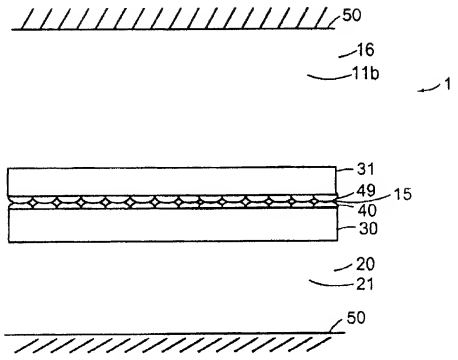


FIG. 5c

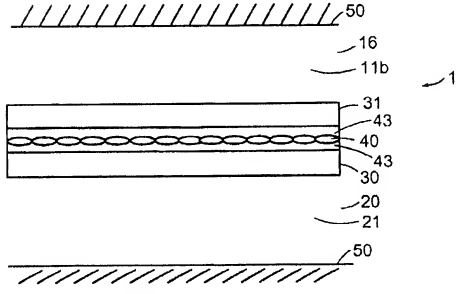


FIG. 5d

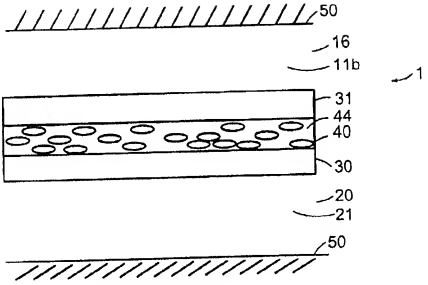


FIG. 5e

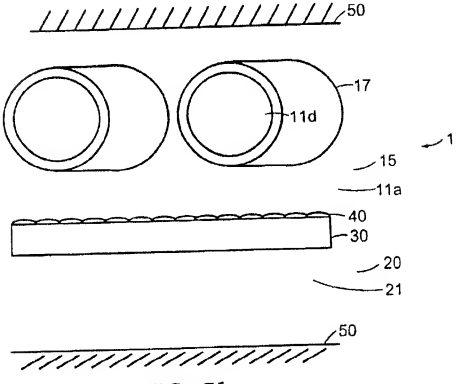


FIG. 5f

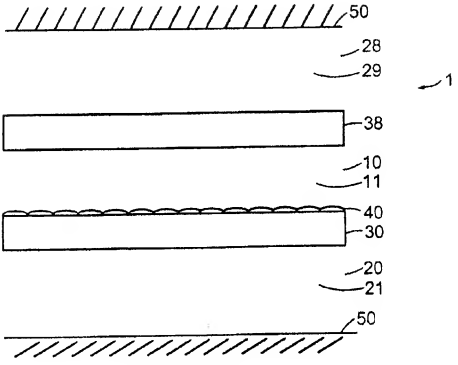


FIG. 6a

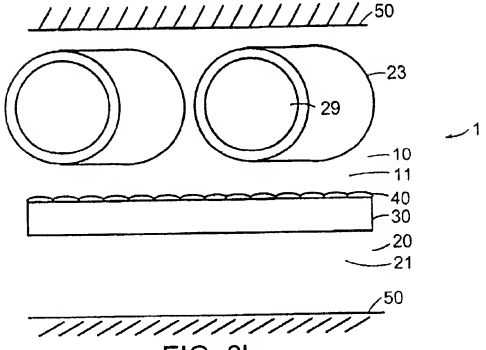


FIG. 6b

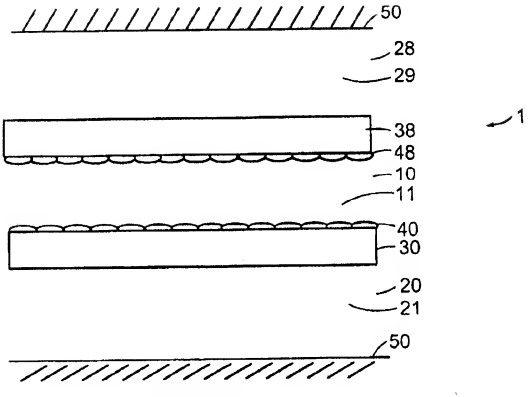


FIG. 6c

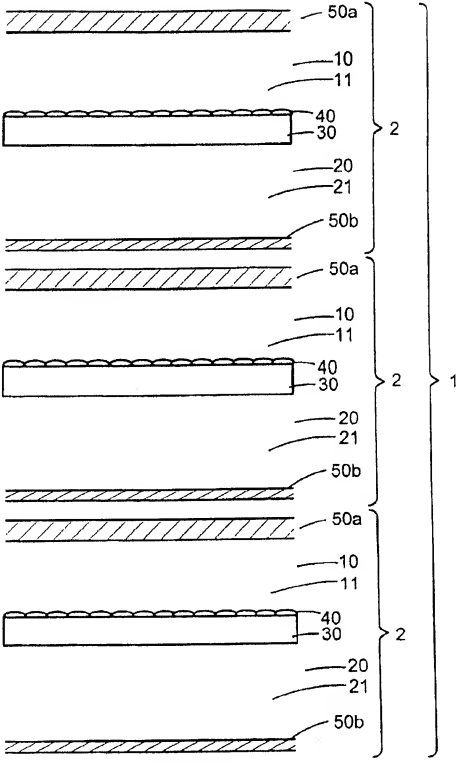

50a
10
11
40
30
2
20
21
50b
50a
10
11
40
30
2
20
21
50b
50a
10
11
40
30
2
20
21
50b
1

FIG. 7a

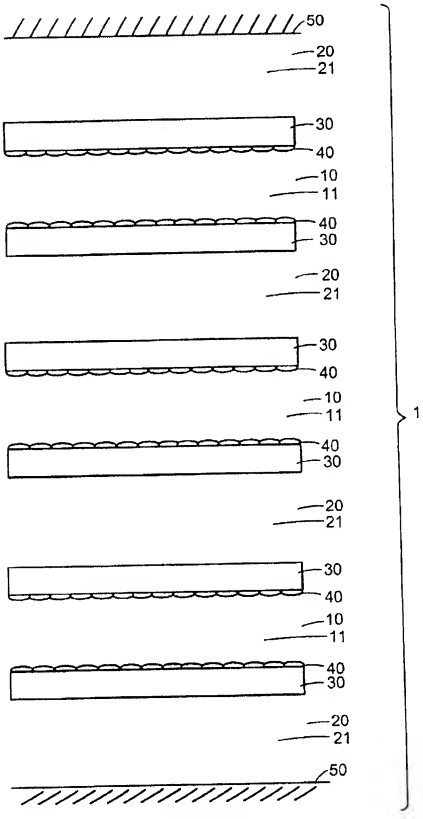

50
20
21
30
40
10
11
40
30
20
21
30
40
10
11
40
30
20
21
30
40
10
11
40
30
20
21
50
1

FIG. 7b

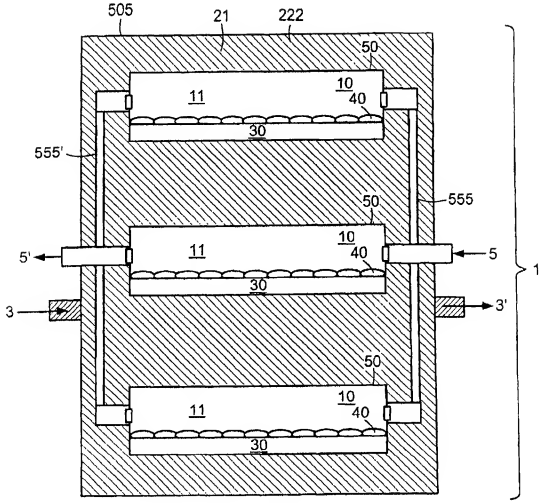


FIG. 8a

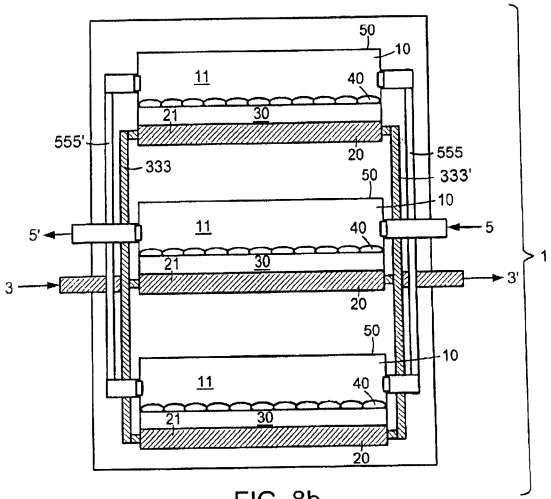

50
10
11
40
21
30
555'
333
20
555
50
333'
10
5'
11
40
5
21
30
1
3
3'
20
50
10
11
40
21
30
20

FIG. 8b

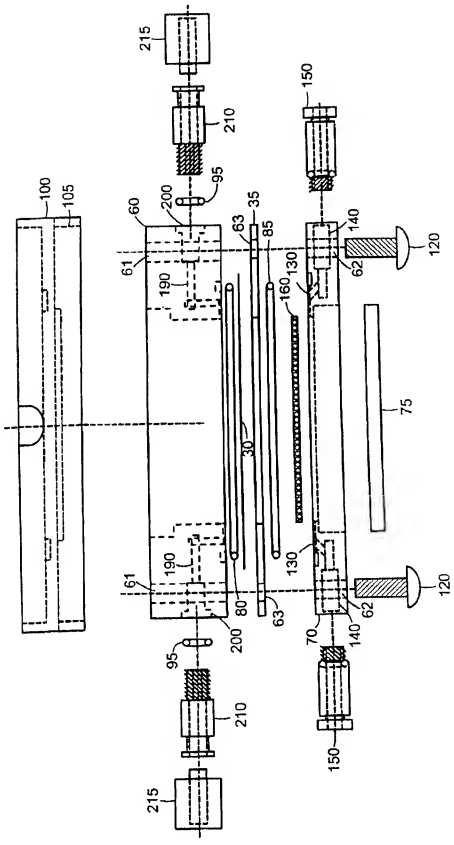

215
210
95
200
60
61
190
63
35
85
30
80
100
105
150
140
120
62
130
160
75
70

FIG. 9A

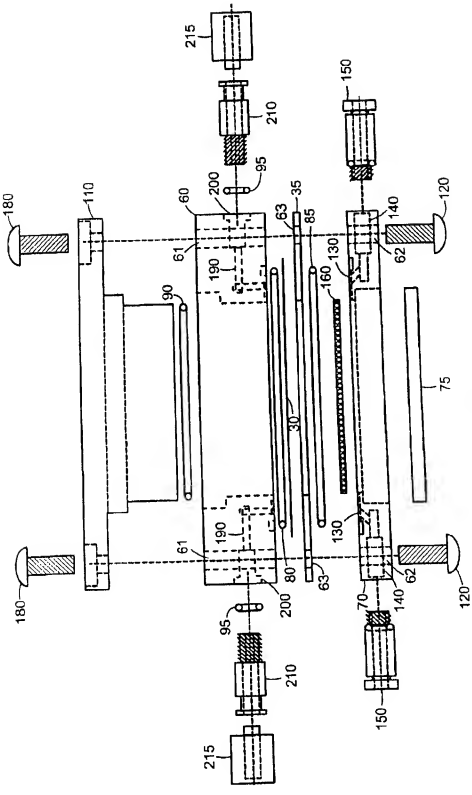

215
210
95
200
60
61
190
90
180
110
63
35
85
150
140
120
62
130
160
75
30
80
70

FIG. 9B

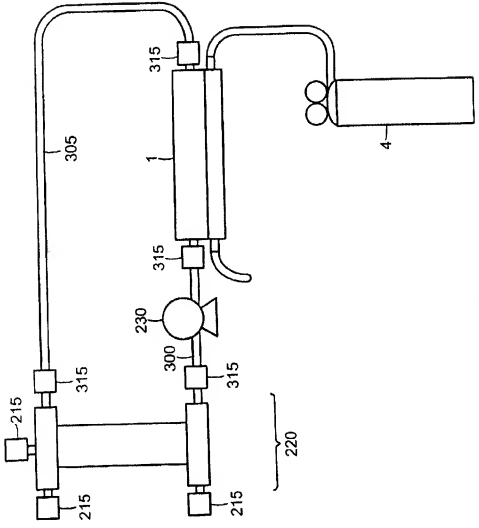


FIG. 10a

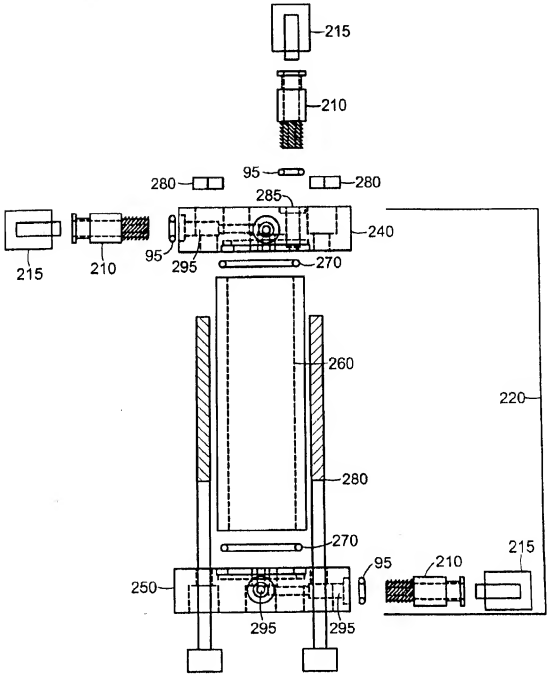

215
210
280
95
280
285
240
215
210
95
295
270
260
220
280
270
215
95
210
250
295
295

FIG. 10B

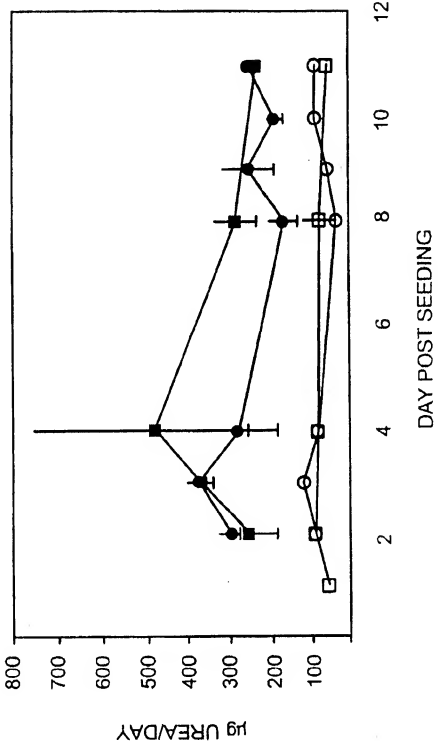
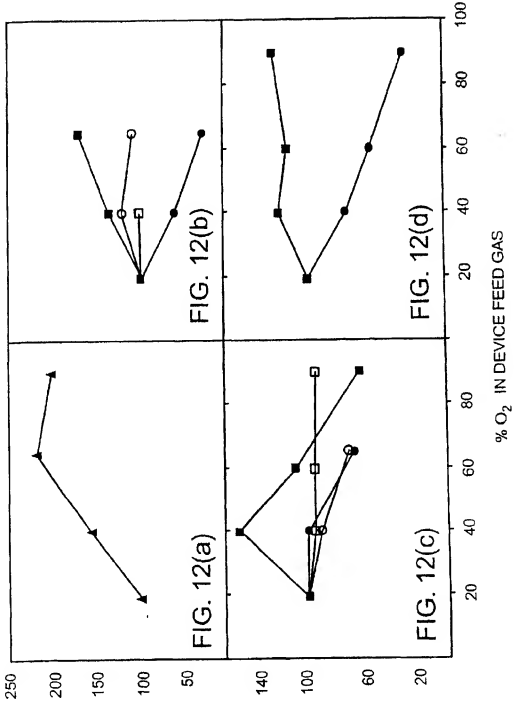

800
700
600
500
400
300
200
100
µg UREA/DAY
2
4
6
8
10
12
DAY POST SEEDING

FIG. 11


250
200
150
100
50
140
100
60
20
%19% O2-CONTROL
FIG. 12(a)
FIG. 12(b)
FIG. 12(c)
FIG. 12(d)
20
40
60
80
100
% O2 IN DEVICE FEED GAS

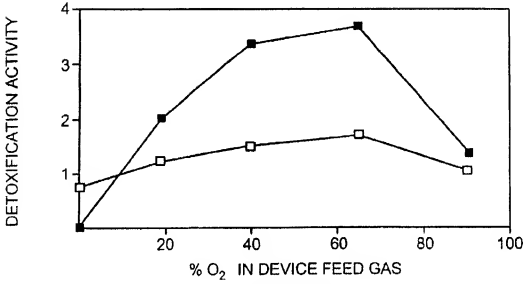

DETOXIFICATION ACTIVITY
4
3
2
1
20
40
60
80
100
% O_2 IN DEVICE FEED GAS

FIG. 13

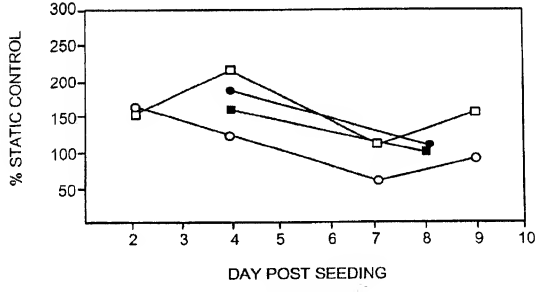

% STATIC CONTROL
300
250
200
150
100
50
2
3
4
5
6
7
8
9
10
DAY POST SEEDING

FIG. 14

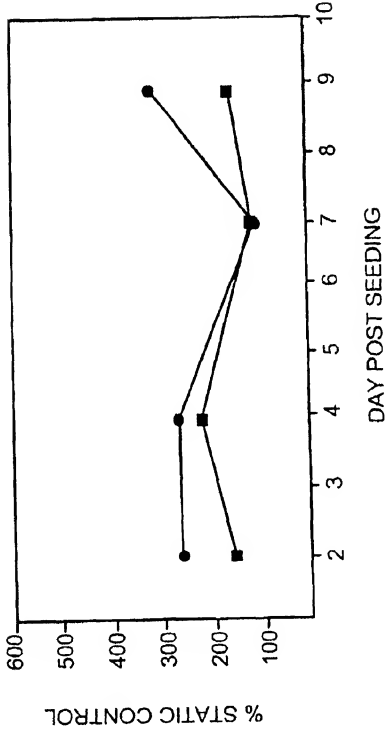

% STATIC CONTROL
600
500
400
300
200
100
2
3
4
5
6
7
8
9
10
DAY POST SEEDING

FIG. 15

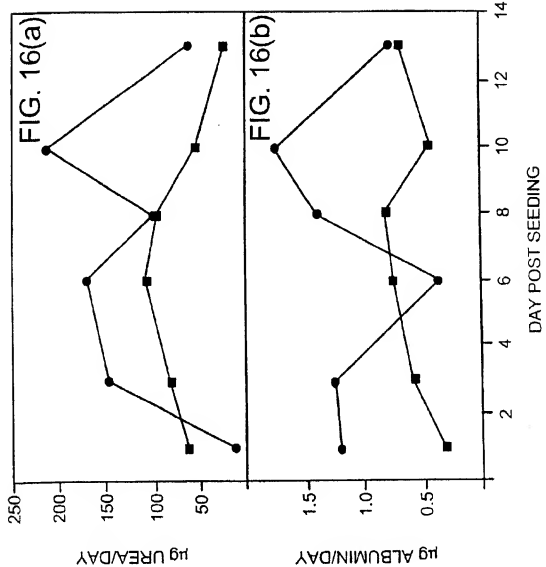


FIG. 16(a)
FIG. 16(b)
μg UREA/DAY
μg ALBUMIN/DAY
250
200
150
100
50
1.5
1.0
0.5
2
4
6
8
10
12
14
DAY POST SEEDING

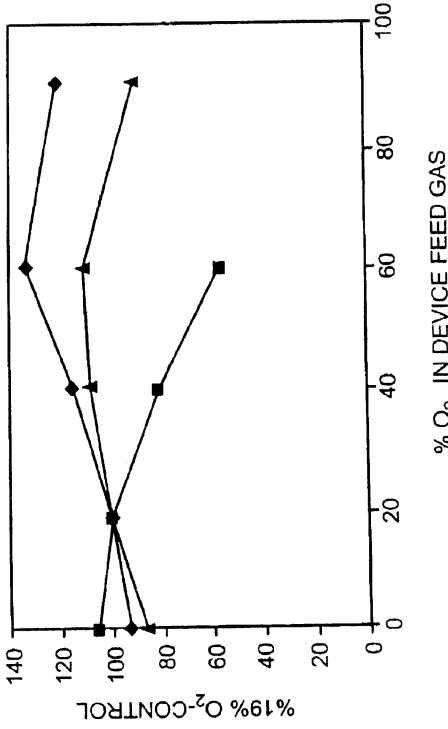

%19% O2-CONTROL
140
120
100
80
60
40
20
0
0
20
40
60
80
100
% O2 IN DEVICE FEED GAS

FIG. 17

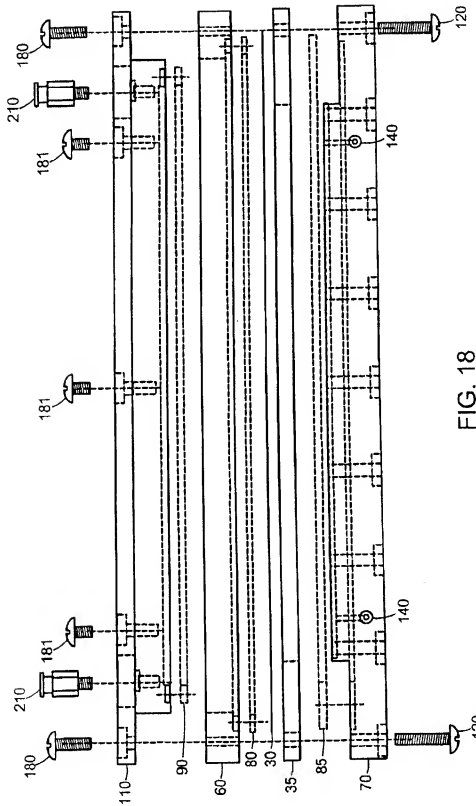

180
210
181
181
181
210
180
110
90
60
80
30
35
85
70
140
140
120
120

FIG. 18

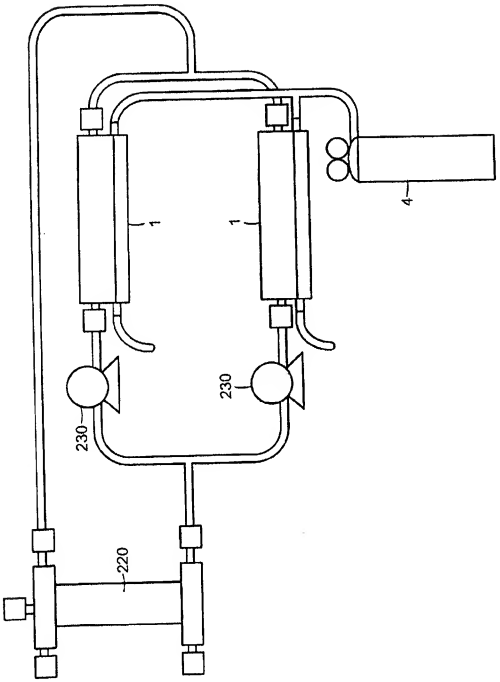


FIG. 19

CELL CULTURE SYSTEMS AND METHODS FOR ORGAN ASSIST DEVICES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Application No. 60/140,125, filed Jun. 21, 1999, U.S. Provisional Application No. 60/140,239, filed on Jun. 21, 1999, and U.S. Provisional Application No. 60/181,634, filed on Feb. 10, 2000. The contents of these applications are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The invention relates to systems and methods of culturing cells in organ assist devices.

BACKGROUND OF THE INVENTION

Over 43,000 Americans die each year from liver disease, making it the tenth leading disease-related cause of death in the U.S. When liver disease progresses to liver failure, the mortality is 80% unless a compatible donor organ is found. As with other organs, there is a critical shortage of donor livers. Over 12,000 patients are currently listed as transplant candidates, but fewer than half that number of donor livers become available each year. Treatment with a liver assist device (LAD) would decrease the mortality associated with liver failure by stabilizing patients so that they are suitable candidates for a transplant, by supporting them until a suitable donor liver becomes available, and/or by preventing deterioration to the point where a liver transplant is required. Improving the pre-operative health of these patients would also increase transplant success, thereby decreasing the frequency of retransplantation and easing the demand for donor organs.

In cases of sudden or hepatic failure, which often occurs as a result of viral infection or toxicity, treatment with a LAD would eliminate the need for a transplant by supporting these individuals until their own livers regenerate. Liver transplantation is currently the most expensive organ transplant procedure. Successful development of a LAD would consequently provide major benefits to the US in reduced deaths and health-care costs.

Extracorporeal devices for temporary liver support have been investigated since the 1960s. Two strategies have been explored in the development of liver assist devices: (1) non-biological devices based on hemoperfusion on sorbents, hemodialysis across selectively-permeable membranes, and plasma exchange (Malchesky, "Non-biological liver support: historic overview," Artif. Organs, 18:342–347, 1994); and (2) biological devices that incorporate cells or cellular components (Yarmush et al., "Assessment of artificial liver support technology," Cell Trans., 1:323–341, 1992).

Non-biological devices have shown only limited efficacy, confirming that synthetic materials cannot replace the range and level of complex metabolic functions normally performed by the liver. On the other hand, a biological LAD in which hepatocytes are seeded on the outer surface of hollow fibers and blood or plasma circulates through the lumen of these fibers was proposed almost 25 years ago by Wolf and colleagues (Wolf et al., "Bilirubin conjugation by an artificial liver composed of cultured cells and synthetic capillaries," Tran. Amer. Soc. Artif. Int. Organs, 21:16–23, 1975).

Current biological LAD designs use the inverse of this concept today. Modern designs are often based on providing

critical liver function by supporting high-density hepatocyte suspensions in hollow fibers, with circulation of blood or plasma outside the fibers. In this design, intermittent extracorporeal liver function is to be provided until the patient recovers through liver regeneration or until a transplant becomes available. However, the hollow fiber design is limited by several factors, including: a) inadequate mass transport, particularly of oxygen, b) lack of understanding of hepatocyte function in an in vitro environment, c) randomized tissue architecture for support of cell viability and function, and d) constraints of void volume on the perfusion circuit for the device.

Hollow fibers have been chosen for LADs on the basis of ready availability rather than demonstrated ability to support hepatocyte function. Perfusion of high-density hepatocyte cultures in hollow fibers has shown a lack of convincing benefit due to, among other reasons, transport limitations that undermine their support of high-density cultures. Such limitations are particularly acute for oxygen, which is required for both basic metabolic function as well as for initial steps in detoxification. Perfusion of oxygenated plasma or medium through or around a network of hollow fibers fails to address this problem because these aqueous liquids are poor carriers for oxygen and the associated distances for transport are relatively large. Modifications to the core hollow-fiber design (e.g., the use of a woven network of three independent sets of capillaries providing integral oxygenation) significantly complicate fabrication and incompletely address underlying transport limitations. They also lack the ability to orient hepatocytes in a more organotypic laminar configuration.

SUMMARY OF THE INVENTION

The invention features modular cell culturing devices comprised of one or more flat-plate modules. The invention is based on the discovery that if the flows of liquid medium and an oxygenated fluid are separated by a gas-permeable, liquid-impermeable membrane, and the cells are grown cultured on the liquid side of the membrane, the device can be used to culture cells with transport of oxygen through the membrane to the cells with independent control of the flow rate of the liquid passing through the device. The new flow-through cell culturing devices can thus be used to culture cells, e.g., hepatocytes, with high levels of cell function in organ, e.g., liver, assist systems, for production of cells, for production of cell-derived products, such as proteins or viruses, or for systems to treat biological liquids to remove toxins, such as ammonia, add cell-synthesized products, or both.

In general, the invention features methods and devices for the culture of cells that provide direct oxygenation of cells through planar, gas-permeable membranes. When the apparatus is seeded with the appropriate cells and is incorporated into a device, the device can be used to treat a patient with an organ, such as the liver, in need of functional assistance.

The invention features methods for culturing cells including: providing a gas-permeable, liquid-impermeable membrane having a first surface and a second surface; seeding cells on the first surface of the gas-permeable, liquid-impermeable membrane; contacting the cells with a nutrient-containing culture medium; providing an oxygenated fluid to the second surface of the gas-permeable, liquid-impermeable membrane at a pressure sufficient to provide transmembrane oxygenation to the cells seeded on the first surface; and culturing the cells under conditions that promote viability and function of the cells.

The device can be seeded with hepatocytes, e.g., porcine, equine, ovine, bovine, rabbit, rat, canine, feline, or murine hepatocytes. Additionally, the device can be seeded with human hepatocytes. The device can be seeded with 2 to 20 billion hepatocytes. The hepatocytes can be seeded directly onto the gas-permeable, liquid-impermeable membrane and then coated with collagen. Alternatively, the gas-permeable, liquid-impermeable membrane can be coated with collagen, and the hepatocytes can be seeded directly onto the collagen-coated membrane. Cells can seeded across the entire membrane from above the membrane.

In one embodiment, the oxygen contained in the oxygenated fluid is at or above the critical partial pressure of oxygen.

In one embodiment, the cells are preserved. The cells can be preserved by cryopreservation, hypothermic storage, or lyophilization.

The gas-permeable, liquid-impermeable membrane material can be made of, e.g., polystyrene, polyolefin, polyethylene, polypropylene, polyvinylidene fluoride, polycarbonate, hydrophobic-treated nylon, polyurethane, polyester, layered styrene-butadiene-styrene/ethyl vinyl acetate/styrene-butadiene-styrene, or layered styrene-butadiene-styrene/polyethylene.

The first surface of the gas-permeable, liquid-impermeable membrane can be treated, e.g., corona treated. In another embodiment, the first surface of the gas-permeable, liquid-impermeable membrane is collagen coated.

In one embodiment, the concentration of oxygen in the oxygenated fluid is between about 0% to about 90% oxygen. Additionally, the concentration of oxygen in the oxygenated fluid can be between about 19% to about 60%, or 40% to about 60%, oxygen. The concentration of oxygen in the oxygenated fluid can be controlled to promote or downregulate cell function.

In one embodiment, the nutrient-containing culture medium is perfused. Additionally, the method can further include filtering blood plasma.

The invention also features a flow-through cell culturing device including a housing with an oxygenated fluid inlet and an oxygenated fluid outlet, a liquid inlet and a liquid outlet, and first and second walls to form a chamber; a gas-permeable, liquid-impermeable membrane arranged between the first and second walls to separate the chamber into an oxygenated fluid compartment comprising an oxygenated fluid entry and an oxygenated fluid exit, and a liquid compartment comprising a liquid entry and liquid exit; and a liquid-permeable membrane arranged between a wall and the gas-permeable, liquid-impermeable membrane to separate the liquid compartment into a cell compartment and a liquid perfusion compartment, wherein the oxygenated fluid inlet and oxygenated fluid outlet are arranged such that oxygenated fluid entering the oxygenated fluid inlet flows into the oxygenated fluid entry and through the oxygenated fluid compartment and exits the oxygenated fluid compartment through the oxygenated fluid exit and the housing through the oxygenated fluid outlet, and wherein the liquid inlet and liquid outlet are arranged such that liquid entering the liquid inlet flows into the liquid entry and through the liquid-perfusion compartment and exits the liquid-perfusion compartment through the liquid exit and the housing through the liquid outlet.

In one embodiment, wherein in use, cells are seeded onto the gas-permeable, liquid-impermeable membrane, and the space between the gas-permeable, liquid-impermeable and liquid-permeable membranes is greater than the size of a cell. In addition, wherein in use, cells can be seeded onto either of the gas-permeable, liquid-impermeable membrane or the liquid-permeable membrane, and the space between the gas-permeable, liquid-impermeable and liquid-permeable membranes is about equal to the size of one cell. Additionally, wherein in use, cells can be seeded onto the gas-permeable, liquid-impermeable membrane, and onto the liquid-permeable membrane, and the space between the gas-permeable, liquid-impermeable and liquid permeable membranes is about equal to the size of two adjacent cells.

The device can further include a liquid-permeable hollow fiber arranged in the liquid compartment. Additionally, the housing can be arranged to enable stacking of one device on top of another device.

The invention also includes a liver assist system including a flow-through cell culturing device of the invention; a first conduit for conducting plasma from a patient to the housing inlet; a second conduit for conducting plasma from the cell culturing device to the patient; and a pump for moving plasma through the conduits and cell culturing device. The system can further include a plasma separator to remove blood cells from whole blood to provide plasma that is passed through the cell culturing device. The system can additionally include a bubble trap, to remove bubbles from the plasma in the first conduit prior to entering the cell culturing device.

The invention also features a liver assist system including a flow-through cell culturing device of the invention; an immunoisolation device; a first conduit for conducting plasma from a patient to an immunoisolation device; a second conduit for conducting plasma from the immunoisolation device to the patient; a third conduit for conducting liquid medium from the cell culturing device to the immunoisolation device; and, a fourth conduit for conducting liquid medium from the immunoisolation device to the patient; and, a pump for moving plasma through the conduits and cell culturing device.

The invention also includes a method of filtering blood plasma. This method includes seeding a flow-through cell culturing device of the invention with hepatocytes; introducing blood plasma into the liquid inlet of the device; supplying an oxygenated fluid into the oxygenated fluid inlet of the device; allowing the oxygenated fluid to flow through the oxygenated fluid compartment and out of the device through the oxygenated fluid outlet; and allowing the blood plasma to flow through the device and exit through the liquid outlet, thereby filtering the blood plasma.

The invention also includes a method for treating a patient in need of liver assist. The method includes attaching the liver assist system of the invention to the blood flow of a patient and treating the patient.

The invention also features a flow-through cell culturing device including a housing with a liquid inlet and a liquid outlet, an oxygenated fluid inlet and an oxygenated fluid outlet, and first and second walls to form a chamber; and a gas-permeable, liquid-impermeable membrane arranged between the walls to separate the chamber into an oxygenated fluid compartment comprising an oxygenated fluid entry and an oxygenated fluid exit, and a liquid compartment comprising a liquid entry and liquid exit, wherein the gas-permeable, liquid-impermeable membrane is seeded with cells, wherein the liquid inlet and liquid outlet are arranged such that biological liquid entering the liquid inlet flows into the liquid entry and through the liquid compartment and exits the liquid compartment through the liquid

exit and the housing through the liquid outlet, and wherein the oxygenated fluid inlet and oxygenated fluid outlet are arranged such that oxygenated fluid entering the oxygenated fluid inlet flows into the oxygenated fluid entry and through the oxygenated fluid compartment and exits the oxygenated fluid compartment through the oxygenated fluid exit and the housing through the oxygenated fluid outlet.

The gas-permeable, liquid-impermeable membrane can be porous or non-porous. The gas-permeable, liquid-impermeable membrane comprises polystyrene, a polyolefin, polyethylene, polypropylene, polyvinylidene fluoride, polyurethane, poly(styrene-butadiene-styrene), poly(ethyl vinylacetate), nylon, silicon rubber, poly (tetrafluoroethylene), or composites, mixtures, or copolymers thereof. The gas-permeable, liquid-impermeable membrane can be surface treated, e.g., with a corona discharge or a coating of extracellular matrix.

In one embodiment, a gel is disposed on the cells. Alternatively, a gel can be disposed on the gas-permeable, liquid-impermeable membrane. The gel can contain cells suspended within said gel.

The invention also features a method of filtering blood plasma including seeding a flow-through cell culturing device of the invention with hepatocytes; introducing blood plasma into the liquid inlet of the device; supplying an oxygenated fluid into the oxygenated fluid inlet of the device; allowing the oxygenated fluid to flow through the oxygenated fluid compartment and out of the device through the oxygenated fluid outlet; and allowing the blood plasma to flow through the device and exit through the liquid outlet, thereby filtering the blood plasma.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The new flow-through cell culturing devices and organ assist systems provide numerous advantages. The new devices allow various cells to be cultured with desirable levels of mass transport of oxygen and other nutrients, waste products, and beneficial products, while potentially reducing detrimental shear stress normally associated with higher levels of media flow. As a result, even relatively shear-sensitive cells such as hepatocytes can be cultured for extended periods of time at relatively low media flow rates with high levels of function. As a consequence, oxygenation and perfusion can be controlled independently. Further, these devices allow direct treatment of surfaces for promotion of cell attachment and function as well as more uniform distribution of cells within the devices in the form of laminar cultures that simulate the in vivo architecture of the liver. These features allow the new flow-through cell culturing devices to be used in organ, e.g., liver assist systems.

Clinical studies have shown that adequate liver function can be maintained in vivo with as little as 10% of the normal cell mass suggesting that an effective LAD would support at least 10^{10} hepatocytes with approximately in vivo levels of function.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic diagram of an extracorporeal liver support system.

FIG. **2** is a schematic diagram of one embodiment of a "two-compartment" cell culturing device of the invention including a gas-permeable, liquid-impermeable membrane separating the two compartments.

FIG. **3** is a graph showing the partial pressure of oxygen in the device feed gas necessary to achieve sufficient oxygenation, under transmembrane oxygenation, to balance the oxygen consumption rate of hepatocytes for a range of cell densities (ρ_{cells}) and for different membrane permeabilities to oxygen and/or cellular rates of oxygen consumption.

FIGS. **4***a* through **4***f* are schematic diagrams of embodiments of "two-compartment" cell culturing devices of the invention including a gas-permeable, liquid-impermeable membrane separating the two compartments.

FIGS. **5***a* through **5***f* are schematic diagrams of embodiments of "three-compartment" cell culturing devices of the invention including separate gas-permeable, liquid-impermeable and liquid-permeable membranes.

FIGS. **6***a* to **6***c* are schematic diagrams of embodiments of multi-compartmented cell culturing device comprising at least two gas-permeable, liquid-impermeable membranes.

FIGS. **7***a* and **7***b* are schematic diagrams of embodiments of the cell culturing device comprising a plurality of stacked compartments.

FIGS. **8***a* and **8***b* are schematic diagrams of embodiments of manifolding for a cell culturing device comprising a plurality of stacked "two-compartment" cell culturing chambers.

FIGS. **9***a* and **9***b* are schematic diagrams of side views of cell culture devices.

FIGS. **10***a* and **10***b* are schematic diagrams of the components of a perfusion circuit for cell culture devices.

FIG. **11** is a graph comparing the synthesis of urea by hepatocytes cultured statically in devices comprising collagen-coated, corona treated 0.002"-thick gas-permeable polystyrene and in tissue-culture dishes.

FIGS. **12***a*, **12***b*, **12***c*, and **12***d* are graphs showing the effects of oxygenation on (**12***a*) attachment of cells one day post seeding, (**12***b*) basal and challenged synthesis of urea, (**12***c*) detoxification activity for alkoxyresorufins, and (**12***d*) metabolism of lidocaine by hepatocytes cultured statically in devices comprising a 0.002"-thick corona-treated, collagen-coated polystyrene gas-permeable membrane.

FIG. **13** is a graph showing the effect of transmembrane oxygenation on detoxification activities for alkoxyresorufin by hepatocytes cultured statically in devices comprising TYVEK® 1073.

FIG. **14** is a graph showing the effect of changes in the volumetric flow rate of medium on synthesis of urea by hepatocytes cultured in perfused devices comprising 0.002"-thick corona-treated, collagen-coated polystyrene.

FIG. **15** is a graph showing the effect of changes in the volume of medium on synthesis of urea by hepatocytes cultured in perfused devices comprising 0.002"-thick corona-treated, collagen-coated polystyrene.

FIGS. **16***a* and **16***b* are graphs showing the effect of changes in the volume of medium on (a) synthesis of urea, and (b) on the secretion of albumin for hepatocytes cultured in perfused devices comprising TYVEK® 1073.

FIG. **17** is a graph showing the effects of transmembrane oxygenation and cell seeding density on ureagenesis for

hepatocytes cultured in perfused devices comprising 0.002"-thick corona-treated, collagen-coated polystyrene.

FIG. **18** is a schematic diagram of a side view of cell culture device sized to treat a rat.

FIG. **19** is a schematic diagram of a pair of cell culture devices arranged in a parallel configuration in a single perfusion circuit.

DETAILED DESCRIPTION

The new cell culturing devices enable the culture of relatively large numbers and high densities of cells, e.g., hepatocytes, in the devices while minimizing total void volume of liquid; allowing precise control, scalability and modularity; and separating medium flow (for supply of nutrients and soluble toxins and/or inducers, and removal of wastes and metabolic byproducts) from oxygenation. The new devices are inherently scalable and modular. The new devices allow separation of oxygenation from flow of biological liquids through the use of a gas-permeable but liquid-impermeable membrane on which the cells are grown or cultured directly.

FIG. **1** shows a schematic diagram of an extracorporeal liver support system in which the new cell culturing devices can be used. The system includes a bioreactor **1** with multiple cartridges **2**. The bioreactor includes an oxygenated fluid inlet **3** for introducing an oxygenated fluid from an oxygenated fluid supply **4**, an oxygenated fluid outlet **3'**, a liquid inlet **5** for introducing a biological liquid, supplied by pump **6** from immunoisolation unit **7**, into the bioreactor, and a liquid outlet **5'** for removing the biological liquid from the bioreactor for return to the immunoisolation unit **7**. Blood from a patient **9** flows via pump **6'** into a plasmapheresis unit **8**, from which a portion of the plasma then flows into the immunoisolation unit **7**, via pump **6"**. Treated plasma flows from the immunoisolation unit **7** and is mixed with blood from the plasmapheresis unit **8** prior to flowing back into the patient **9**.

The new cell culturing devices contain one or more two-compartment cartridges (or units). FIG. **2** shows one embodiment of a two-compartment cartridge that includes a chamber **1** having impermeable walls **50** and a gas-permeable, liquid-impermeable membrane **30** separating the chamber into two regions, or compartments, a first compartment **10**, and a second compartment **20**. The first compartment **10** is defined by one of the impermeable walls **50**, the side walls of the chamber, and the gas-permeable, liquid impermeable membrane **30**. The first compartment **10** is a "liquid compartment" containing a biological liquid **11**, such as cell culture medium, a balanced salt solution, blood, or plasma, and cells **40**. The cells **40** are substantially cultured on the membrane **30** and in contact with the biological liquid **11**.

Walls are typically composed of cell-compatible plastic such as polystyrene and co-polymers that are thick to make them impermeable to liquids and gases, metals or composites thereof such as aluminum, stainless steel and other alloys. These materials can be used for other device components including fittings and manifolds.

The second compartment **20** is a "oxygenated fluid compartment" and is defined by the second of the impermeable walls **50**, the side walls of the chamber, and the gas-permeable, liquid-impermeable membrane **30**. An oxygenated fluid **21**, typically a gas but also possibly a liquid, capable of carrying oxygen or an oxygenated gas and allowing the transport of oxygen through the gas-permeable, liquid-impermeable membrane **30**, flows through the compartment **20**. Oxygen is available to the cells in the liquid compartment from the oxygenated fluid compartment via the gas-permeable, liquid impermeable membrane **30**. Oxygenated fluids can be gases or liquids and can include air, oxygen-enriched air, oxygen gas, mixtures of oxygen and other gases such as carbon dioxide, nitrogen, argon, helium and other gases commonly found in nature; liquids such as fluorocarbons, perfluorinated liquids, and aqueous solutions containing natural or synthetic hemoglobin or equivalents.

Although FIG. **2** depicts the chamber **1** divided such that the liquid compartment **10** is above the gas-permeable, liquid-impermeable membrane **30** and the oxygenated fluid compartment **20** is below the membrane, the chamber can be oriented in any direction as long as the membrane divides the chamber into two compartments. Preferably the height of each compartment is constant (although not necessarily the same for both compartments); however, the height of each compartment can be non-uniform, e.g., varying along the length and/or width of the compartment.

The biological liquid **11** in the first (liquid) compartment **10** supplies the cells **40** with basic nutrients for cell culture and carries away metabolites. The biological liquid also supplies the cells with toxins, aminated molecules, and other biological waste products to be metabolized and carries away detoxified products, secreted factors, and proteins For in vitro culture of cells this biological liquid preferably is a medium designed for cell culture. The biological liquid is preferably supplied to and removed from the compartment interior through an opening. More preferably there are at least two openings (not shown in FIG. **2**), one opening serving as an inlet opening to supply biological liquid from a biological liquid source and another opening serving as an outlet to discharge or drain the biological liquid. The openings communicate between the interior and exterior of the liquid compartment **10** by a port or manifold. Ports can be connected with other liquid compartments of other bioreactor units or cartridges in parallel, in series, or both, to create a flow circuit or loop for the perfusion of the biological liquid. The addition of other ports can serve as vents for air displacement during filling or as a means of draining the compartment when the other ports are attached to those of another unit.

The oxygenated fluid in the second (gas) compartment **20** is preferably supplied to and removed from the compartment interior through an opening. More preferably there are at least two openings (not shown in FIG. **2**), one opening serving as an inlet opening to supply oxygenated fluid from an oxygenated fluid source **4** and another opening serving as an outlet to discharge or vent oxygenated fluid. The openings communicate between the interior and exterior of the oxygenated fluid compartment **20** by a port or manifold. Again, ports can be connected with other oxygenated fluid compartments of other bioreactor units or cartridges in parallel, in series, or both, to create a flow circuit or loop for the oxygenated fluid. Other ports for venting may also be added to the compartment wall.

The membrane **30** is gas-permeable and liquid-impermeable. Cells **40** may attach on any surface inside the liquid compartment, but it is preferred that the cell mass be substantially cultured on the membrane and that the membrane serves as a cell culture substrate. Cells exchange gas such as oxygen with the oxygenated fluid through the membrane. The membrane preferably is in a planar, flat-sheet configuration and extends in a plane to separate the liquid compartment **10** and the oxygenated fluid compartment **20**. The membrane is gas-permeable to allow transport of oxygen and possibly other gases from the oxygenated

fluid to the cells and from the cells to the oxygenated fluid. The membrane must be impermeable to liquid under pressures encountered in operation but permeable to gas in the range from about 0.1 mL/m^2/day to about 1000 L/m^2/day. The membrane must also be able to be sterilized, resistant to puncture, ripping, and wrinkling, and be able to be handled during manufacture of the device.

Membrane materials of one or more layers having the following characteristics are suitable for use in the invention: relatively permeable to oxygen and at least partially impermeable to water in the absence of large pressure differences across the membrane **30**, relatively non-cytotoxic to cells on at least one side (such that the attachment and function of the cells is not limited by the material or that the material can be surface treated on one side such that the attachment and function of the cells is not limited by this surface-treated side of the material), and relatively non-degrading in the presence of the oxygenated fluid **21** and/or biological liquid **11**. For double-sided materials the side facing the liquid compartment **10** must be relatively non-cytotoxic and relatively non-degrading in the presence of the biological liquid and the side facing the oxygenated fluid compartment **20** must be relatively non-degrading in the presence of the oxygenated fluid. Membrane materials having these characteristics can be easily obtained commercially or prepared using standard techniques.

Specific membrane materials suitable for use in the invention include single layers and multi-laminate composites of non-porous or microporous materials such as non-porous polystyrene, including 0.002-in thick polystyrene such as POLYFLEX® (from Plastics Suppliers); microporous polyolefin; microporous high-density polyethylene (HDPE), such as TYVEK®, particularly TYVEK® 1073 (DuPont); microporous polypropylene; microporous polyvinylidene fluoride; track-etched polycarbonate (with relatively small pores and no-wetted); hydrophobic-treated nylon; polyurethane; microporous polyester (with hydrophobic pores); other inorganic polymers, such as microporous inorganic polymers and non-porous silicone rubbers; co-extruded polystyrene and polyethylene; a three-layered co-extruded film of styrene-butadiene-styrene/ethyl vinyl acetate/styrene-butadiene-styrene (SBS/EVA/SBS); and a two-layered co-extruded film of styrene-butadiene-styrene/polyethylene (SBS/PE).

Oxygenation of cells **40** through the gas-permeable, liquid-impermeable membrane **30** requires that either the membrane itself be self-supporting (e.g., possess sufficient mechanical stiffness to withstand gravity, the weight of the membrane, the weight of the biological liquid if the biological liquid is oriented above the membrane, and any applied pressure differences across the membrane) or that the membrane be combined with or laminated onto a stiffer support material. This support material must be either sufficiently permeable, porous, or spatially distributed such that it presents no additional significant limitations to gas transport, particularly for oxygen, and also has the requisite mechanical properties. For example, the use of impermeable posts relatively widely spaced to support the membrane can satisfy these requirements.

In the culture of some cell types, oxygenation is by the cell culture medium. However, some cell types, such as hepatocytes, have oxygen requirements that are characteristically high compared to other cell types. The function of viable hepatocytes can also depend on oxygen tension. Another consideration which intensifies this type of problem in a cell culturing or organ assist device is the need to incorporate relatively large numbers and high densities of cells into the device while limiting volume of biological liquid.

The rate at which oxygen is consumed by the cells must be balanced by the transport of oxygen to the cells, either by transport of oxygen across the membrane or through the flowing biological liquid. The kinetics of oxygen consumption by the cells is given by the oxygen uptake rate (OUR), typically described in terms of Michaelis-Menten kinetics and expressed as moles of oxygen consumed per cell per unit time, multiplied by the cell number. The rate of transport of oxygen across the membrane is given by the product of the volumetric flow of oxygen per unit of partial pressure difference (expressed as volume of oxygen per unit area per unit time per unit partial pressure difference), the partial pressure difference across the membrane, and the projected area of the membrane. The rate of transport of oxygen through the biological liquid is given by the negative of the product of the diffusivity of oxygen in the biological fluid, the gradient in the concentration of oxygen perpendicular to the membrane at the surface of the membrane where the cells are cultured on, and the projected area of the membrane.

Minimum levels of oxygenation of cells maintained in the cell culturing device can be determined by balancing the transport of oxygen into the device with the rate of consumption of oxygen by cells within the device. For devices operated statically, i.e., without introducing new biological liquid into the device, the only mechanism of transport for oxygen is transmembrane. The rate of transmembrane transport is given by the product of the flux of oxygen across the membrane and the planar area of the membrane; the flux is itself a product of the permeability of the membrane, P_m, and the difference in concentration of oxygen across the membrane. The rate of consumption of oxygen is given by the OUR.

For devices operated statically the governing equation balancing transport and consumption is:

$$pO_{2,c} = RT\left(\frac{OUR}{P_m}\right),$$

where $pO_{2,c}$ is the critical partial pressure of oxygen for oxygenation, R is the gas constant, and T is temperature. Partial pressures of oxygen greater than this $pO_{2,c}$ provide sufficient oxygenation; partial pressures of oxygen below this $pO_{2,c}$ provide insufficient oxygenation. This equation can be used to design and operate such cell culturing devices **1** in the absence of flow of the biological liquid **11**.

The governing equation for oxygenation is plotted in FIG. **3** for simulated data for devices comprising 0.002 in-thick polystyrene, available as Polyflex® from Plastics Suppliers (Columbus, Ohio), for which P_m is on the order of 10^6 mL/m^2-day-atm, and operated at physiological temperatures (310 K) and for cellular rates of consumption of oxygen of 1×10^{-16}, 5×10^{-16}, and 1×10^{-15} moles/cell-s. These values for consumption of oxygen are based on data for primary porcine hepatocytes presented in Balis et al., Metabolic Engineering, 1:1–14 (1999). These curves will shift towards the top and left when membranes with lower permeabilities to oxygen are used and towards the bottom and right when membranes with higher permeabilities to oxygen are used.

In contrast, when oxygenation is supplied to cells on a gas-impermeable support through the layer of quiescent biological liquid bathing them and a gas-permeable membrane in contact with that layer of biological liquid, the governing equation balancing transport and consumption of oxygen is:

$$pO^{*}_{2,d} = RT\left(\frac{OUR}{P_m}\right)\left(1+\frac{hP_m}{SRTD}\right),$$

where h is the thickness of the layer of the biological liquid, S is the solubility of oxygen in the biological liquid, and D is the diffusivity of oxygen in the biological liquid. Because the transport resistance to oxygen will always be greater for this configuration, assuming a membrane with an identical P_m, this second configuration will always result in less oxygenation for a given concentration of oxygen.

The governing equations also will depend on the perfusion of medium. However, the basic trends between the two governing equations will not change with direction, volumetric flow rate, or any other characteristic of perfusion.

For example, greater control of oxygenation will always be possible, for membranes with identical permeabilities to oxygen, for direct transmembrane oxygenation of cells compared to sequential transmembrane and then transmedium oxygenation of cells, regardless of the rate of perfusion, provided that the directionality of oxygenation per se does not effect the cells.

Various other specific embodiments of two-compartment cell culturing devices with a gas-permeable, liquid-impermeable membrane will now be described. These embodiments are depicted schematically in FIGS. **4***a*–**4***i*.

FIG. **4***a* shows an alternate basic configuration for a two-compartment cell culturing device **1** comprising a cell compartment **10** containing a biological liquid **11**; an oxygenated fluid compartment **20** containing an oxygenated fluid **21**; a gas-permeable, liquid-impermeable membrane **30** with surface **41**; cells **40**; and rigid, impermeable walls **50**. The liquid compartment **10** and the oxygenated fluid compartment **20** are separated by the gas-permeable, liquid-impermeable membrane **30**, surface treated on the side of the membrane facing the liquid compartment. The cells **40** are cultured on the surface **41** while contacting the biological liquid **11** in the liquid compartment and are able to access oxygen through the gas-permeable, liquid-impermeable membrane **30** from an oxygen source that supplies oxygenated fluid **21** to the oxygenated fluid compartment **20**.

The surface **41** may be advantageous or, as with some cells that do not adhere well, necessary, to induce cell adhesion and promote desired cell function. This surface, which may include one or more coating materials, is applied to at least a portion of the membrane and may extend into the interior of the membrane from the side facing the liquid compartment **10**. Coating materials that can be applied to the membrane include, but are not limited to: biological coatings such as collagen and other extracellular matrix components, including fibronectin, preparations of extracted biological matrices, and proteoglycans; fibrin; RGD-containing and similar peptides; biosynthetic coatings chemically or biologically synthesized, and/or some other materials that will induce cell adhesion and/or desired cell functions. A preferred coating is collagen, more preferably collagen type I. These coatings may either passively adsorb to the membrane or chemically react (such as by covalent grafting) with the membrane. The membrane may need to be treated with one or more coatings or layers of coatings to make cells adhere and function; these coatings may be identical or dissimilar in composition and/or concentration.

Still another preferred means for improving cell compatibility to the membrane is to provide it with a collagen coating. Coatings, distinguished from bulk gels, are essentially two-dimensional as they are molecular in thickness and are dry on the membrane surface while bulk gels have bulk, three-dimensional thickness and are hydrated. Multiple surface treatments may be applied to the membrane in sequence, for example, treatment with corona discharge followed by coating of extracellular matrix, such as collagen, preferably with collagen type I.

Another method for treating the membrane **30** to improve cell adhesion is a physical surface treatment, such as corona discharge in the presence of an oxygen-bearing gas (e.g., air). Corona discharge incorporates oxygen atoms into the exposed surface such that it is oxidized, more hydrophilic, and sometimes charged. The extent of this treatment is such that the material now possesses a surface free energy (measured in terms of dynes/cm^2 by wettability) and other chemical characteristics similar to those characteristics typically associated with tissue-culture dishes. This treatment is conducted for a period of time sufficient to achieve modification of the exposed planar surface to this extent (or higher or lower, depending on whether the cells respond more favorably to a surface with a different free energy), but without significantly compromising the properties of the underlying base material itself. Multiple surface treatments may be applied in sequence, for example, treatment with corona discharge followed by coating with extracellular matrix, such as collagen, preferably with collagen type I.

Corona discharge treatment typically modifies only the surface chemistry of a material or membrane without altering its physical topography. Corona discharge involves exposing the surface to a high-frequency electrical discharge in the presence of air or other oxygen-bearing gas, such that oxygen atoms are incorporated into the surface of the material such that it is now oxidized. This treatment increases the surface fee energy and hydrophilicity of plastic, paper, and metalized films. Systems to apply a corona discharge to materials are commercially available from companies such as Corotec Corp., Farmington, Conn. These systems consist of a high voltage transformer and web of electrodes designed to apply up to kilowatt levels of energy to the surface at frequencies as high 40 kHz. Exposing oxygen to a high voltage electrical discharge generates ozone, which chemically reacts with the surface to incorporate oxygen into it. This treatment may be carried out at room temperature under environmental conditions typically found in chemical and biological laboratories. Operating conditions for corona discharge may vary with the material being surface modified and the extent to which the surface free energy is to be increased.

In another embodiment, the cells are sandwiched between the gas-permeable, liquid-impermeable membrane **30** and a bulk gel comprising extracellular matrix components. FIG. **4***b* shows a cell culturing device **1** comprising a liquid compartment **10** containing a biological liquid **11**; an oxygenated fluid compartment **20** containing an oxygenated fluid **21**; a gas-permeable, liquid-impermeable membrane **30**; cells **40**; a bulk gel **42**, and rigid, impermeable walls **50**. The liquid compartment **10** and the oxygenated fluid compartment **20** are separated by the gas-permeable, liquid-impermeable membrane **30** on which the cells **40** are cultured on the side of the membrane facing the liquid compartment. A bulk gel **42** is disposed over the cells on the side of the cells facing away from the membrane. Cells are provided with nutrients from the biological liquid **11** through the bulk gel **42** and can access oxygen through the gas-permeable, liquid-impermeable membrane **30** from an oxygen source **4** that supplies oxygenated fluid **21** to the oxygenated fluid compartment **20**.

In another embodiment shown in FIG. **4***c*, the cells are sandwiched between two bulk gels, such as two bulk col-

lagen gels **43** as described in International PCT Application Publication No. WO 96/34087 and in U.S. Pat. No. 5,602,026. In particular, FIG. **4***c* shows a cell culturing device **1** comprising a liquid compartment **10** containing a biological liquid **11**; an oxygenated fluid compartment **20** containing an oxygenated fluid **21**; a gas-permeable, liquid-impermeable membrane **30**; cells **40**; a two-layer gel **43**, and rigid, impermeable walls **50**. The liquid compartment **10** and the oxygenated fluid compartment **20** are separated by the gas-permeable, liquid-impermeable membrane **30** on which the cells **40** are cultured (on the side of the membrane facing the liquid compartment) deposited between the layers of a two-layer gel **43**. Cells **40** are provided with nutrients from the biological liquid **11** through the gel **43**, and can access oxygen through the gas-permeable, liquid-impermeable membrane **30** from an oxygen source **4** that supplies oxygenated fluid to the oxygenated fluid compartment **20**.

Another useful method for binding cells to the membrane is by using a matrix material. FIG. **4***d* shows another embodiment of a cell culturing device **1** comprising a liquid compartment **10** containing biological liquid **11**; an oxygenated fluid compartment **20** containing an oxygenated fluid **21**; a gas-permeable, liquid-impermeable membrane **30**; cells **40** within a cell-supporting lattice or scaffold **44**; and rigid, impermeable walls **50**. This lattice preferably is collagen based, although it also may be based on alternative natural extracellular matrix materials and/or synthetic polymers and blends of polymers. Collagen and other extracellular matrix components can be formed in an uncontracted or contracted lattice comprising collagen to suspend cells in a matrix. When the material is collagen, the lattice with cells is deposited by casting a mixture of acid-solubilized collagen, a pH neutralizing solution, and a cell suspension. This cast gels to form a cell-containing collagen lattice **44**. Methods for producing cell-containing collagen lattices, particularly contracted collagen lattices, are previously described in U.S. Pat. Nos. 4,485,096, 5,106,949, and 5,536,656.

Alternatively, the lattice may be a porous, natural or synthetic scaffold wherein the cells are cultured within the scaffold and the scaffold contacts the gas-permeable membrane cells. The porous support either is first applied to the membrane and the cells then seeded into the porous scaffold or first seeded with cells and then applied to the membrane. Examples of porous supports include scaffolds comprising extracellular matrix components, such as collagen sponges, dense fibrillar collagen membranes, and processed tissue membranes. Synthetic porous supports include polyurethane foams.

FIGS. **4***e* and **4***f* show two additional embodiments of a cell culturing device **1** comprising a liquid compartment **10** containing a biological liquid **11**; an oxygenated fluid compartment **20** containing an oxygenated fluid **21**; a gas-permeable, liquid-impermeable membrane **30**; cells **40** within a cell-supporting lattice **44**; a second layer of cells **45**; and rigid, impermeable walls **50**. FIG. **4***e* depicts an embodiment in which the second layer of cells **45** is on the side of the lattice **44** facing the liquid compartment **10**. FIG. **4***f* depicts a second embodiment in which the second layer of cells **45** is between the lattice **44** and the membrane **30**. The second layer of cells may be the same type or different from the cells within the lattice. An embodiment consisting of a combination of the arrangement of cells in the embodiments shown in FIGS. **4***e* and **4***f* also can be realized.

Because some cells have attachment or functional properties which can be adversely affected by direct contact with a flowing biological liquid and are shear sensitive, in some applications it may be desirable to place a liquid-permeable membrane between the flowing biological liquid and the cells to limit hydrodynamic interactions. In this arrangement an additional compartment, a cell compartment, containing the cells is located between the liquid compartment and oxygenated fluid compartment.

FIG. **5***a* shows one embodiment of a three-compartment cell culturing device **1** having impermeable walls **50**, a gas-permeable, liquid-impermeable membrane **30** separating a first compartment **20** from a second compartment **15**, and at least one liquid-permeable membrane **31** separating the second compartment **15** from a third compartment **16**. The first compartment **20** is the "oxygenated fluid" compartment and is defined by one of the impermeable walls **50**, the side walls of the chamber, and the gas-permeable, liquid-impermeable membrane **30**. The second compartment **15** is the "cell" compartment and is defined by the side walls of the chamber, the gas-permeable, liquid-impermeable membrane **30**, and the liquid-permeable membrane **31**. The third compartment **16** is the "liquid-perfusion" compartment and is defined by the remaining impermeable wall **50**, the side walls of the chamber, and the liquid-permeable membrane **31**.

Although FIG. **5***a* depicts the chamber **1** divided such that the liquid-perfusion compartment **16** is above the one or more liquid-permeable membranes **31** and the oxygenated fluid compartment **20** is below the gas-permeable, liquid-impermeable membrane **30**, the chamber can be oriented in any direction as long as the cell compartment **15** intervenes between the liquid-perfusion and oxygenated fluid compartments and the gas-permeable, liquid-impermeable membrane **30** is located between the oxygenated fluid and cell compartments and one or more liquid-permeable membranes **31** are located between the cell and liquid-perfusion compartments.

The liquid-perfusion compartment **16** contains a biological liquid **11***b*, such as cell culture medium, a balanced solution, blood, or plasma. The cell compartment **15** contains both cells **40** substantially cultured on the gas-permeable, liquid-impermeable membrane **30** as well as a biological liquid **11***a* that may be the same or different from the biological liquid **11***b* in the liquid perfusion compartment **16**. The biological liquids **11***a* and **11***b* are in liquid contact through the intervening one or more liquid-permeable membranes **31**. The biological liquid **11***a* supplies the cells **40** with basic nutrients for cell culture, toxins, aminated molecules, and other biological waste products to be metabolized and carries away cell metabolites, detoxified products, secreted factors, and proteins. These molecules are transported across the liquid-permeable membrane **31** to and from the biological liquid **11***a*.

The cells **40** in the cell compartment **15** are substantially not in contact with the liquid-permeable membrane **31**. The gap filled by the volume of the cells and the biological liquid **11***a* in the cell compartment preferably is uniform in thickness but may be nonuniform. This gap is at least the height of the layer of cells **40** attached to the gas-permeable, liquid-impermeable membrane **30**, such that at least some biological liquid **11***a* intervenes between the cells **40** and the face of the liquid-permeable membrane **31** facing the cells. This gap is maintained by one or more spacers.

The biological liquid **11***a* in the cell compartment **15** flows very slowly or is static; its flow is substantially unaffected by the flow of the biological liquid **11***b* in the liquid-perfusion compartment **16**. The biological liquid **11***a* preferentially is initially supplied to the cell compartment **15** during filling and is free to exchange with the biological

liquid **11***b* across the liquid-permeable membrane **31**. One or more ports can serve as vents for air displacement during filling and/or as means of draining the cell compartment **15** during operation. These other ports may also be manifolded to ports from cell compartments of other bioreactors units in parallel, in series, or both for venting and drainage.

The biological liquid **11***b* is preferentially supplied to and removed from the interior of the liquid-perfusion compartment **16** through an opening. More preferably there are at least two openings (not shown in FIG. **5***a*), one opening serving as an inlet opening to supply biological liquid **11***b* from a biological liquid source and another opening serving as an outlet to discharge or drain the biological liquid **11***b*. The openings communicate between the interior and exterior of the liquid-perfusion compartment **16** by a port or manifold. Ports can be connected with other liquid-perfusion compartments of other bioreactor units in parallel, in series, or both to create a flow circuit or loop for the biological liquid **11***b*. The additional of other ports can serve as vents for air displacement during filling or as a means of draining the liquid-perfusion compartment **16** when the other ports are attached to those of another unit.

An oxygenated fluid **21**, typically a gas but also possibly a liquid capable of carrying oxygen or an oxygenated gas and allowing the transport of oxygen through the gas-permeable, liquid-impermeable membrane **30**, flows through the oxygenated fluid compartment **20**. Oxygen is available to the cells **40** in the cell compartment **15** via the gas-permeable, liquid-impermeable membrane **30**. The oxygenated fluid in the oxygenated fluid compartment is preferentially supplied to and removed from the compartment interior through an opening. More preferably there are at least two openings (not shown in FIG. **5***a*), one opening serving as an inlet opening to supply oxygenated fluid from an oxygenated fluid source and another opening serving as an outlet to discharge or vent oxygenated fluid. The openings communicate between the interior and exterior of the oxygenated fluid compartment **20** by a port or manifold. Again, ports can be connected with other oxygenated fluid compartments of other bioreactor units in parallel, in series, or both to create a flow circuit or loop for the oxygenated fluid. Other ports for venting may also be added to the compartment wall.

Various other specific embodiments of three-compartment cell culturing devices with separate gas-permeable, liquid-impermeable and liquid-permeable membranes will now be described. These embodiments are depicted schematically in FIGS. **5***b–f*. FIG. **5***b* shows one embodiment in which the cells **40** are in direct contact with both the gas-permeable, liquid-impermeable membrane **30** and the liquid-permeable membrane **31**. In this embodiment, the cell compartment **15** is reduced in size to a minimal volume, specifically the size of the cells **40** in the cell compartment, when the liquid-permeable membrane **31** contacts the cells growing on the gas-permeable, liquid-impermeable membrane **30**.

Another alternative embodiment is shown in FIG. **5***c*, in which one layer of cells **40** is attached to the gas-permeable, liquid-impermeable membrane **30** and a second layer of cells **49** is attached to the liquid-permeable membrane **31**, to form a cell bilayer or multilayer culture. This embodiment comprises at least two layers of cells. Additional layers of cells may intervene between the layers of cells **40** and **49**.

In another embodiment shown in FIG. **5***d*, the cells **40** are sandwiched between two bulk collagen gels **43**. In particular, FIG. **5***d* shows a cell culturing device comprising a liquid-perfusion compartment **16** containing a biological liquid **11***b*, such as cell culture medium, a balanced solution, or plasma. The cell compartment **15** contains a first bulk collagen gel **43** disposed on a gas-permeable, liquid-impermeable membrane **30**; one or more layers of cells **40** seeded on the gel; a second bulk collagen gel **43** disposed upon the cells; and a liquid-permeable membrane **31** contacting the second gel. The biological liquid **11***b* is in liquid contact through the intervening one or more liquid-permeable membranes **31**. The biological liquid **11***b* supplies to, and removes from, the cells **40** components related to cell culture that are transported across the bulk collagen gels **43**, the liquid-permeable membrane **31** to and from the biological liquid **11***b*.

FIG. **5***e* illustrates another embodiment in which a cell-supporting lattice **43** containing cells **40** is sandwiched between the gas-permeable, liquid-impermeable membrane **30** and the liquid-permeable membrane **31**. The cell-supporting lattice can be a collagen lattice or can be interchanged with a porous matrix containing cells as described elsewhere herein.

For the embodiments depicted in FIGS. **5***b–5e*, a spacer may be added to maintain the height of the cell compartment **15**. For the embodiment of FIG. **5***b* this spacer may be omitted if the cells are first seeded onto one of the two membranes **30** and **31** and then the membranes brought together with the cells sandwiched between the two membranes. For the embodiment of FIG. **5***c* this spacer may be omitted if each cell layer **40** is seeded first onto its adjacent membrane and then the cell-seeded membranes brought together.

In another embodiment, hollow fibers can be used as the liquid-perfusion compartment **16**. A variation of this embodiment is shown in FIG. **5***f*, in which cells **40** are attached to the planar, gas-permeable, liquid-impermeable membrane **30** and hollow fibers **17** extending in a plane above the cells and containing biological liquid **11***d* are located in cell compartment **15**. Biological liquid **11***a* in cell compartment **15** can be static or can flow. Biological liquid **11***d* (which can be the same or different from biological liquid **11***a*) preferably flows within the hollow fibers **17**. The hollow fibers **17** are made of a liquid-permeable material, much like the liquid-permeable membrane **31** described herein. In this way, nutrients in biological liquid **11***d* can be transported throughout the device at a flow rate that is independent of the flow rate of biological liquid **11***a* in the cell compartment (which is preferably low to reduce sheer stress on cells **40**). The orientation of the hollow fibers **17** may be parallel, anti-parallel, or orthogonal to the direction of flow in the oxygenated fluid compartment **20**.

To effectively separate the biological liquid **11***d* in the liquid-perfusion compartment **16** from the biological liquid **11***a* in the cell compartment **15**, the liquid-permeable membrane **31** or hollow fibers **17** have a pore size large enough to allow for the transport of large molecule nutrients, toxins, factors, metabolites, and secreted proteins, across the membrane but small enough to prevent cell growth through the membrane. Suitable materials for the liquid-permeable membrane include, but are not limited to: cellulosic membranes; porous poly(sulfone) and poly(ether sulfone) membranes; porous untreated nylon and other polyamide membranes; porous polyesters; porous glass; perforated stainless steel; porous (i.e., track-etched) polycarbonate (wetted with PVP, ethanol, or other wetting agents), porous polyvinyl chloride, perforated polydimethylsiloxane; and porous ceramics. Other membrane materials having these characteristics for use in the invention can be selected from commercially available materials or can be prepared using standard techniques.

The flow of oxygenated fluids can be static, in any one direction, or in multiple directions. In relation to oxygenated fluid flow, flow of biological liquids may be in any direction: the same lateral direction, contralateral, or perpendicular, or any combination thereof. For maximal mass transfer preferably the directions of flow are contralateral, e.g. in opposite directions for the biological liquid and oxygenated fluid. For bioreactor units or cartridges connected in parallel, in series, or both the relative directions of flow in each cartridge can be the same or different. The flow of biological liquids and/or oxygenated fluids can be oscillating or time-dependent. Also, the volumetric flow rates of the biological liquids and oxygenated fluids are independent of each other.

The bioreactor unit of the invention includes at least one compartment containing cells and at least one compartment containing oxygenated fluid. The compartments are in a flat, planar arrangement and have a common shared plane between them. A single unit or cartridge can comprise one compartment containing cells and one compartment containing oxygenated fluid housed individually or together in a stacked configuration, with the units separated by a rigid, impermeable member. Because of the unitary nature of the cell culture cartridges or units, the bioreactor is scalable with the addition of surface area and volume to the compartments or the addition of units. In the case where additional units are added, it is preferred that the compartments communicate via the ports to allow flow of biological liquid or medium or oxygenated fluid between them.

FIG. **6***a* illustrates an embodiment of the cell culturing device comprising one bioreactor unit with two planar gas-permeable, liquid-impermeable membranes, **30** and **38**, respectively. Only one of the membranes, **30**, is seeded with cells **40** on its side facing the liquid compartment **10** and contacting the biological liquid **11**. This embodiment features two oxygenated fluid compartments, **20** and **28**, respectively. The oxygenated fluids in the oxygenated fluid compartments **20** and **28**, **21** and **29**, respectively, may be the same or different, may be flowing in the same or different directions, and may be flowing at the same or different flow rates. This embodiment allows simultaneous direct oxygenation of adherent cells **40** through transmembrane transport of oxygen across the membrane **30** while also providing additional direct oxygenation of the flowing biological liquid **11** through transmembrane transport of oxygen across the membrane **38**.

Another embodiment of the three-compartment cell culturing device featuring one liquid and two oxygenated fluid compartments is illustrated in FIG. **6***b*. One gas-permeable, liquid-impermeable membrane **30** is planar and seeded with cells **40** on its side facing the liquid compartment **10** and contacting the biological liquid **11**. The second oxygenated fluid compartment **23** is formed by hollow fiber placed in a plane through the liquid compartment **10** and containing the oxygenated fluid **29**. The oxygenated fluids **21** (contained in the oxygenated fluid compartment **20**) and **29** may be the same or different, may be flowing in the same or different directions, and may be flowing at the same or different flow rates. This embodiment provides direct oxygenation of the flowing biological liquid **11** through transmembrane transport of oxygen across the hollow fibers **23** in addition to direct oxygenation of adherent cells **40** through transmembrane transport of oxygen across the membrane **30**.

FIG. **6***c* illustrates a third embodiment of the three-compartment cell culturing device featuring one liquid and two oxygenated fluid compartments. This embodiment comprises one bioreactor unit in which the number of cells **40** contacting the flowing biological liquid **11** is increased by contacting the biological liquid with a second layer of cells **48** supported on the second gas-permeable, liquid-impermeable, substantially planar membrane **38**. The directions of flow in oxygenated fluid compartment **20**, oxygenated fluid compartment **28**, and in the liquid compartment **10** may be the same lateral direction, contralateral, or perpendicular, or any combination thereof; one or both of the oxygenated fluids **21** and **29** may be static. The two layers of cells **40** and **48** may be at the same or different densities and may be the same or different types of cells. This embodiment allows increasing the treatment capacity of a single bioreactor unit without altering the number of cells per unit area of gas-permeable, liquid-impermeable membrane or changing the volume of biological liquid perfused through the system.

The cells **40** in the embodiments depicted in FIGS. **6***a* and **6***c* are substantially in contact with only one gas-permeable, liquid-impermeable membrane **30**. The gap filled by the volume of the cells and the biological liquid **11** in the liquid compartment preferably is uniform in thickness but may be nonuniform. This gap is at least the height of the layer of cells **40** attached to the membrane **30**, such that at least some biological liquid **11** intervenes between the cells and the face of the liquid-permeable membrane facing the cells. There is no absolute limit on the height of this gap for this invention. This gap may be maintained by a spacer.

One embodiment of a bioreactor comprising several stacked cartridges is shown in FIG. **7***a*, in which bioreactor **1** includes three stacked cartridges **2** (see also FIG. 1). Each cartridge **2** includes a housing including upper and lower walls **50***a* and **50***b*, respectively. Each cartridge includes a liquid compartment **10**, through which the biological liquid **11** flows, and an oxygenated fluid compartment **20**, through which the oxygenated fluid **21** flows. The two compartments of each cartridge or unit **2** are separated by a gas-permeable, liquid-impermeable membrane **30**. The cells **40** are cultured on the membrane **30** on the side of the membrane facing the liquid compartment. The flow of oxygenated fluids and biological liquids can be in any direction: the same lateral direction, contralateral, or perpendicular, or any combination thereof. The number of compartments can vary up to 100 or more of each type of compartment. Further, the composition (e.g., percentage oxygen) and volumetric flow rates of the individual flows of oxygenated fluids **21** may be identical or differ among the oxygenated fluid compartments **20**. Similarly, the composition (e.g., concentration of cytokines) and volumetric flow rates of the individual flows of biological liquid **11** may be identical or differ among the liquid compartments **10**.

An alternative embodiment in which a bioreactor **1** comprises a plurality of stacked gas-permeable, liquid-impermeable membranes, with cells cultured on the side of the membrane facing towards a biological liquid and away from an oxygenated fluid, is shown in FIG. **7***b*. In this embodiment the bioreactor **1** comprises one large chamber having an inlet and an outlet for flow of the oxygenated fluid **21** through the bioreactor **1** and an inlet and an outlet for flow of the biological liquid **11** through the entire bioreactor **1**. In this embodiment the bioreactor **1** does not contain individual cartridges but instead contains a plurality of stacked gas-permeable, liquid-impermeable membranes **30** (six in FIG. **7***b*), to create alternating cell compartments **10** (three in FIG. **7***b*) containing biological liquid **11** and oxygenated fluid compartments **20** (four in FIG. **7***b*) containing oxygenated fluid **21**. Cells **40** are cultured on the side of each membrane **30** contacting the biological liquid **11**. The number of compartments can vary up to 100 or more of

each type of compartment. As indicated for the embodiment depicted in FIG. 7*a*, the compositions and flow of biological liquid **11** and oxygenated fluid **21** in each liquid and oxygenated fluid compartment, respectively, may be the same or different.

The inlet for oxygenated fluid to the bioreactor can be connected to a first manifold that distributes the flow of oxygenated fluid evenly or unevenly to each of the plurality of oxygenated fluid compartments to deliver oxygen and other gases to the oxygenated fluid compartments for gas exchange across the gas-permeable, liquid-impermeable membranes. After passage through the multiple oxygenated fluid compartments (in parallel), oxygenated fluid is collected in a second manifold common to all the oxygenated fluid compartments and then directed out of the bioreactor through an outlet for oxygenated fluid. Similarly, the inlet for biological liquid is connected to a manifold that distributes the flow of a biological liquid evenly to each of the plurality of liquid compartments to expose cells in the liquid compartments to the biological liquid. After passage through the multiple compartments (again, in parallel), the biological liquid is collected in a common manifold and is directed to an outlet to circulate in the flow loop for biological liquid.

The manifolds for this multi-compartment cell culturing device preferably are connected to their associated compartments by detachable connectors. These connectors allow easy installation and possible replacement of individual connections. Alternatively, the manifolds may be, if desired, permanently connected to each associated compartment.

A specific embodiment of such a manifolded bioreactor **1** is shown in FIG. 8*a*, in which the bioreactor **1** has a plurality of stacked cartridges **2** (three in FIG. 8*a*), each comprising a liquid compartment **10**, a gas-permeable, liquid-impermeable membrane **30**, cells **40**, and a rigid, impermeable housing **50**. The bioreactor **1** also comprises a common oxygenated fluid compartment **222**, oxygenated fluid inlet **3**; oxygenated fluid outlet **3'**; liquid inlet **5**; liquid outlet **5'**, liquid inlet manifold **555**, and liquid outlet manifold **555'**. Liquid inlet manifold **555** conducts biological liquid from liquid inlet **5** to the liquid compartment **10** of each cartridge **2**. Liquid outlet manifold **555'** conducts biological liquid to liquid outlet **5'** from the liquid compartment **10** of each cartridge **2**. The addition of other ports for each liquid compartment can serve as vents for air displacement during filling or as a means of draining each liquid compartment individually. Oxygenated fluid inlet **3** conducts oxygenated fluid into the common oxygenated fluid compartment **222** of the bioreactor **1** outside of the cartridges **2**, where the oxygenated fluid contacts the gas-permeable, liquid-impermeable membrane **30**. The oxygenated fluid is conducted out of the bioreactor vian oxygenated fluid outlet **3'**. Other ports for venting oxygenated fluids may also be added to the impermeable wall **505** of the bioreactor **1**. The number of compartments can vary up to 100 or more of each type of compartment.

FIG. 8*b* shows an alternative configuration in which a bioreactor **1** has a plurality of stacked cartridges **2** (three in this figure), each comprising a liquid compartment **10**, an oxygenated fluid compartment **20**, a gas-permeable, liquid-impermeable membrane **30**; cells **40**, and a rigid, impermeable housing **50**. The bioreactor **1** also comprises an oxygenated fluid inlet **3**; oxygenated fluid outlet **3'**, liquid inlet **5**; liquid outlet **5'**; liquid inlet manifold **555**, and liquid outlet manifold **555'**; oxygenated fluid inlet manifold **333**; and oxygenated fluid outlet manifold **333'**. Liquid inlet manifold **555** conducts biological liquid **11** from liquid inlet **5** to the liquid compartment **10** of each cartridge **2**. Liquid outlet manifold **555'** conducts biological liquid **11** to liquid outlet **5'** from the liquid compartment **10** of each cartridge **2**. The addition of other ports for each liquid compartment can serve as vents for air displacement during filling or as a means of draining each liquid compartment individually. Oxygenated fluid inlet manifold **333** conducts oxygenated fluids from oxygenated fluid inlet **3** to the oxygenated fluid compartment **20** of each cartridge **2**. Oxygenated fluid outlet manifold **333'** conducts oxygenated fluids to oxygenated fluid outlet **3'** from the oxygenated fluid compartment **20** of each cartridge **2**. Other ports for venting oxygenated fluids may also be added to each oxygenated fluid compartment **20**. The number of compartments can vary up to 100 or more of each type of compartment.

In still another embodiment, the cells are separated from the flowing biological liquid by one or more intervening liquid-permeable membranes, such that the liquid compartment now becomes separate liquid-perfusion and cell compartments, which are either completely or incompletely divided by the one or more intervening liquid-permeable membranes. Any number of alternating compartments may be employed and are not bound by the chamber housing as shown in FIG. **7***b*. The chamber housing is made of impermeable material.

The bioreactor is seeded with functional cells; more preferably, the seeded cells function together to simulate the types and levels of function possible for cells in an organ. Cells can either grow in the bioreactor, remain stable in number, or switch between modes of growth and numerical stability. Cells also can either maintain their previous phenotype or change phenotype upon culture in the bioreactor. The bioreactor is to be used as an in vitro culture system and its oxygenated fluid and liquid circuits make it suitable for use as an organ assist device to treat a patient in need of organ assistance. The device is seeded with organ cells that are cultured to function similarly as in the organ from which the cells were derived.

One source of cells for the bioreactor is a mammalian organ. When this organ is the liver, the cells that are cultured for use in a liver assist device comprise hepatocytes, the principal cells of the liver which are capable of fulfilling the functional requirements typically associated with the liver when placed in an appropriate chemical and structural environment. Other cells present in liver may also be included in the device, such as endothelial cells; Ito cells; Kupfer cells, a specialized macrophage-like cell; and fibroblasts. A co-culture of hepatocytes with one or more of these or other types of cells may be desirable in a LAD. For bioreactors comprising a plurality of units each unit may be seeded with the same numbers of cells and/or combinations of types of cells or different numbers and/or combinations.

Given the limited availability of human hepatocytes, non-human sources can be used in the invention. Cells from other mammals including, but not limited to, equine, canine, porcine, bovine, ovine, and murine sources can be used. Cell donors can vary in development and age, sex, species, weight, and size. Cells may be derived from donor tissues of embryos, neonates, or older individuals including adults. Embryonic progenitor cells such as parenchymal or mesenchymal stem cells can be used in the invention and induced to differentiate to develop into the desired tissue. In addition, mixtures of cells from different cell strains, mixtures of normal and genetically modified cells, or mixtures of cells from two or more species or tissue sources may be used.

One source of hepatocytes for use in the invention is porcine liver. The best current estimates of the number of viable hepatocytes for each clinical LAD is approximately

10^{10} cells, roughly the number of cells from a small (20 lb.) pig liver. An alternative source of cells is by culturing either cells previously obtained from a mammalian organ or by culturing cells that have been previously cultured such that they exist as a cell line. Cells for use in the invention may be normal or genetically engineered by spontaneous, chemical, or viral transfection. Recombinant or genetically engineered cells can be created for immortality, reduced allogenicity, or differentiated hepatocyte function. Procedures for genetically engineering cells are generally known in the art, and are described in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989).

Cells are seeded from fresh, processed tissue, from cells cultured previously in vitro, thawed from cryopreserved tissue, or some combination thereof. Prior to seeding cells are suspended in a seeding medium. In the new methods and devices the cells are seeded onto the gas-permeable, liquid-impermeable membrane. Prior to seeding cells the gas-permeable, liquid-impermeable membrane is treated, if desired, and the liquid compartment drained of most but not necessarily all of its liquid contents. It also is possible to replace some of the liquid contents with, e.g., a biological liquid, prior to seeding cells. These transfers of liquid may be conducted either by draining through one or more of the ports for the liquid compartment, by removing the impermeable wall contacting the liquid compartment and dispensing and aspirating liquid directly into the liquid compartment through the opening created by the removal of the above-mentioned impermeable wall, or a combination thereof.

Multiple embodiments are possible for seeding of cells. In one embodiment the cells are introduced by first removing the impermeable wall contacting the liquid compartment and dispensing (with a manual or automated pipette) cells, in a liquid suspension or suspended in a natural or synthetic polymer matrix, onto the gas-permeable, liquid-impermeable membrane. In an alternative embodiment cells are introduced by first making sure at least two ports for the liquid compartment are opened and then allowing the suspension of cells to flow into the liquid compartment. For either of these embodiments the cells are allowed to attach and establish functionality on the membrane. The seeding medium then may be drained (by any of the methods described in the above paragraph for removal and addition of liquids from the liquid compartment) and replenished with a biological liquid.

For a bioreactor containing a plurality of culture units or cartridges, each unit or cartridge may be seeded with cells either together with the other units in the assembled state by one of the above embodiments or seeded with cells separately as individual units not bundled together with manifolds and then subsequently manifolded together into a complete bioreactor system. The time at which seeded units are assembled together in this latter embodiment may be soon after seeding or after allowing further establishment of cultures.

Once cultures have become established and the bioreactor is functional as a liver assist device, it is used to treat a patient in need of liver assistance, as shown in FIG. **1**. As shown in FIG. **1** and as described herein, the liver assist device of the invention comprises a bioreactor **1** having an inlet **3** and an outlet **3'** for the supply of oxygenated fluid and an inlet **5** and an outlet **5'** for the supply of biological liquid. The inlet **3** for the supply of oxygenated fluid to the oxygenated fluid compartments is fed by an oxygenated fluid supply **4**. The oxygenated fluid outlet **3'** is preferably vented to the atmosphere or a collection vessel. Also associated with the oxygenated fluid supply **4** are controllers to monitor the oxygenated fluid mixture, pressure, and flow rates (not shown).

The inlet **5** and outlet **5'** for the biological liquid are connected in a closed loop. It is desirable, but not necessary, to have in the loop for the biological liquid an immunoisolation unit **7** to isolate the blood flow of the patient from the bioreactor. The bioreactor **1** may be associated with a plasmapheresis unit **8** to separate the treated patient's plasma from blood to make blood detoxification more efficient. Within the flow of the biological liquid are various pumps **6**, **6'**, and **6"** to ensure the flow of medium or biological liquid through the bioreactor and the immunoisolation unit and pH, temperature, and flow sensors (not shown).

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Examples 1, 2, and 14 describe new cell culturing devices. Examples 3 through 8 describe assays used to measure hepatocellular function. Examples 9 through 14 demonstrate certain embodiments of the invention.

Example 1

Cell Culture Device—Static Arrangement

The ability of the new cell culturing device to support the function of hepatocytes in vitro was evaluated by constructing a laboratory-scale device **1** based on this configuration. The device, as depicted in FIGS. *9a* and *9b*, comprised an assembly of upper **60** and lower **70** housings, a gas-permeable, liquid-impermeable membrane **30**, a frame **35**, a window **75** for the lower housing, a set of O-rings **80**, **85**, **90**, and **95**, a cover **100**, a cap **110**, and ancillary screws and fittings **120**, **150**, **180**, **210**, and **215**. The membrane used typically was either Polyflex® (Plastics Suppliers, Inc., Columbus, Ohio), a 0.002"-thick film of polystyrene, or TYVEK® 1073 (E.I. du Pont de Nemours and Co, Inc., Wilmington, Del.), a microporous non-woven membrane composed of USP Class VI polyolefin, although other materials were also used in some studies. The membrane was epoxied onto one face of the frame **35**, a 0.0625"-thick aluminum annulus with an axisymmetric 2.122"-diameter through-hole, to form a membrane/frame assembly **400** prior to use.

A set of four symmetrically-spaced stainless steel #10-32×½" truss-headed screws **120** bolted the opposing 4"-diameter upper **60** (0.750"-thick) and lower **70** (0.315"-thick) polycarbonate housings together to sandwich the membrane/frame assembly **400**, with the membrane/assembly oriented with the membrane **30** facing the upper housing. Through-holes **63** in the frame **35** allowed insertion of the screws **120**. through counter-bored clearance holes **62** in the lower housing **70** into tapped holes in the upper housing **60**. A liquid-tight seal was formed between the upper face of the membrane/frame assembly and the lower face of the upper housing using a #039 O-ring **80** seated in an axisymmetric groove in the upper housing. Similarly, a gas-tight seal was formed between the lower face of the membrane/frame assembly and the upper face of the lower housing using a second #039 O-ring **85** seated in an axisymmetric groove in the lower housing. With these components the minimum diameter of the membrane was 2.9" to match the outer diameter of the O-rings, although membranes with larger diameters could be used by cutting away

sections of the membrane to prevent interference with the path of the screws.

An axisymmetric 2.125"-diameter, 0.062"-deep chamber **30** for gas beneath the membrane was formed by securing a 2.330"-diameter, 0.125"-thick polycarbonate window **75** into an axisymmetric thru-hole in the lower housing **70**. Two channels **130** connected to tapped holes **140** fitted with #10-32×⅛" tube OD Legris fittings with integral O-rings to provide ports for external access to this chamber. Additional mechanical support of the membrane could be provided by placing in this chamber a highly porous 2.0"-diameter, 0.06"-thick block **160**.

Two methods were used to form a 2.125"-diameter chamber **10** holding liquid **11** bathing cells **40** attached to the membrane **30** in an axisymmetric 2.125"-diameter thru-hole in the upper housing **60**. In one method (depicted in FIG. **9***a*) a 0.750"-deep chamber **170**, used when seeding cells onto the membrane, was formed by placement of a 4.216"-diameter polycarbonate cover **100** onto the upper face of the upper housing. This cover, which was intended to allow aseptic transportation of the assembled device after seeding with cells and stacking of multiple devices, had 0.25"-high rims **105** along the perimeter of both its upper and lower faces to prevent spillage of liquid out of the chamber and penetration of external contaminants into the chamber.

In the second method (depicted in FIG. **9***b*) a 0.062"-deep chamber **10**, used after removal of the liquid containing the suspension for seeding cells, was formed by securing a 4.000"-diameter polycarbonate cap **110** into the through-hole in the upper housing **60**. A liquid-tight seal between the cap and upper housing was formed using a #020 O-ring **90** and four symmetrically spaced stainless steel #10-32×½" truss-headed screws **180** which fastened into tapped holes on the upper face of the upper housing. Two channels **190** connected to tapped holes **200**, fitted with #10-32×¼" 316 stainless steel female luer-lock fittings **210** with #006 O-rings **95**, provided ports for external access to these chambers. These ports were plugged for static cultures using 316 stainless steel male luer-lock plugs **215**.

All assembly steps, unless otherwise noted, were conducted in a biological safety cabinet and occurred after sterilizing all parts (other than the membrane/frame assembly **400** and the fittings **150** for the lower housing **110**) by autoclaving or other proven treatment (e.g., gamma irradiation or exposure to an oxidizing gas such as ethylene oxide, peracetic acid, and/or hydrogen peroxide). All materials were handled with either sterile tweezers or gloves within the cabinet. Some membranes were used without sterilization for relatively short durations of a 1–3 days or less; other membranes were either epoxied onto frames and gamma irradiated prior to installation (e.g., Polyflex®) or gamma irradiated without frames prior to installation (e.g., TYVEK® 1073).

The initial steps in assembly consisted of mating the upper **60** and lower **70** housings, the #039 O-rings **80** and **85**, the membrane/frame assembly **400**, the threaded fittings **150** and **210**, and the #006 O-rings **95** and tightening the four screws **120** holding these parts together. Next, the fittings were tightened and plugs **215** for the fittings for the upper housing affixed. The cover **100** then was installed and the device **1** stored under aseptic conditions until use.

Primary hepatocytes **40** in complete medium (Williams E medium supplemented with 4.5 g/L glucose, 0.5 U/mL bovine insulin, 7 ng/mL glucagon, 7.5 μg/mL hydrocortisone, 10 mM HEPES, 20 ng/mL EGF, 20 mM glutamine, 10 IU penicillin, and 10 μg streptomycin) with 1% new-born calf serum (NBCS) were obtained from porcine donors with the following procedure.

Hepatocytes were isolated from livers of Yorkshire/Hampshire crossbred pigs, obtained from E M Parsons (Hadley, Mass.), weighing 8.3±3.0 kg. Heparin (Elkins-Sinn, Cherry Hill, N.J.) was administered intravenously at 0.5 mg/kg and donors anesthetized with a mixture of Telazol (7–10 mg/kg, Fort Dodge Laboratories, Fort Dodge, La.) and Rompun (5 mg/kg, Miles, Inc., Shawnee, Mission, Kans.). Plane of anesthesia was maintained with isoflurane gas. All procedures were performed in compliance with ACUC guidelines.

Cells **40** were isolated using a modification of the Seglen method which has been described previously (P Seglen, Preparation of isolated rat liver cells, In Method in Cell Biology (D M Prescott, et al.), Vol. 13, Academic Press (New York, N.Y.), 1976). Briefly, the exposed liver was cannulated and perfused in situ with cold Lactated Ringers (Baxter, Deerfield, Ill.) at 20 mL/min before excision. The liver was quickly warmed and perfused with 0.2% EDTA at 37° C. This was followed by perfusion of 1 mg/mL collegians (Life Technologies, Grand Island, N.Y.) at 37° C. until digestion appeared complete (mean digestion 22+4 min). Further digestion was stopped with the addition of cold Hank's buffered saline solution (BioWhittaker, Walkerville, Md.) supplemented with 10% NBCS (Cyclone, Logan Utah). Undigested tissue and gall balder were excised and the remainder of the tissue passed sequentially through 200 and 100 micron stainless steel sieves (Fisher Scientific, Pittsburgh, Pa.), respectively. The cell suspension was washed twice and the cell pellet resuspended in culture media. Viability was determined by Trypan blue exclusion.

The cells were then seeded onto the membrane. In some studies the membrane **30** was pre-coated with a sterile 4 mL volume solution of 40 μg/mL Type I collagen in water for 45 minutes, followed by aspiration of this solution and rinsing with an equal volume of sterile phosphate-buffered saline (PBS), prior to seeding of cells. A suspension of cells in medium **11** was evenly suspended by swirling the receiver containing the cells and triturating the suspension multiple times with a sterile pipette prior to seeding. For most studies cells were seeded at an initial density of 2×106 cells per device **1**. In some studies, however, higher densities were examined (see Example 13). The cover **100** in the device **1** was removed, 4 mL of the cell suspension pipetted onto the membrane **30**, the device agitated carefully to distribute the liquid evenly onto the surface of the membrane, and the cover replaced. The cell-seeded device was transferred to an incubator (held at 37° C. and 85% relative humidity) where the gas chamber **20** was connected, through one of the ports **150** in the lower housing **70**, to a gas tank **4** supplying 10% CO_2 and a concentration of oxygen ranging from 0 to 90% at 2–5 psi and no greater than 1.0 mL/min.

After approximately 18–24 hours the device was removed from the incubator, placed back in the biological safety cabinet, cover **100** removed, and the medium **11** aspirated using a sterile Pasteur pipette. The cap **110** with associated #020 O-ring **90** then was placed onto the thru-hole in the upper housing **60** and secured in place using the four screws **180**. Approximately 2.7 mL of fresh medium **11** was introduced into the liquid chamber **10** by removing the luer-lock plugs **215**, transferring in medium at a rate of 1.5 mL/min with the ports oriented vertically (such that all air bubbles were removed from the chamber), and closing the ports by reinstalling the luer-lock plugs. The device then was transferred back to the incubator and reconnected as above to the oxygenated fluid supply **4**. Devices were sampled subsequently by disconnection of the oxygenated fluid supply, transfer from the incubator to a biological safety cabinet,

removal of the luer-lock plugs, and draining of the liquid contents into a small collection vial. This procedure resulted in a device 1 that allowed culture of cells **40** on the membrane **30** with direct transmembrane oxygenation and independent perfusion.

Example 2

Cell Culture Device—Perfused Arrangement

The apparatus of Example 1 also was adapted to study the function of hepatocytes in vitro under closed-loop perfusion of medium. The apparatus was assembled and handled as described in Example 1 through installation of the cap **110**. A simple flow circuit, as depicted in FIGS. **10***a* and **10***b*, was assembled aseptically to the device in a biological safety cabinet. Components for this circuit (illustrated schematically in FIG. **5***a*) comprised a cell-seeded device **1** (with installed cap **110** as described in Example 1), a reservoir **220**, a pump **230**, connecting tubings **300** and **305**, and luer-lock fittings **215** and **315**. All components were sterilized by autoclaving (or equivalent treatment) prior to assembly except the cell-seeded device (which had been handled aseptically). Prior to connecting the cell-seeded device the male luer-lock plugs **215** were removed from the luer-lock fittings **210**.

FIG. **10***b* depicts the reservoir, which functioned also as a bubble trap, comprising a polycarbonate top **240** and bottom **250** (each 2.00" in diameter and 0.500" thick), a 3.00"-tall×0.75"-ID×1.00"-OD polycarbonate tube **260**, a pair of #019 O-rings **270**, 3 metal screws and bolts **280**, and 5 #10-32×¼" 316 stainless steel female luer-lock fittings **210** with #006 O-rings **95**. The O-rings **270** sat in 0.785"-ID×0.965"-OD×0.052"-deep axisymmetric grooves at the bottom of 0.75"-ID×1.00"-OD×0.062"-deep recesses (to align the tube) in the upper and lower faces of the bottom and top, respectively. Liquid-tight seals were formed between the tube and top and bottom, respectively, by seating the tube in these grooves after installation of the O-rings **270** and tightening of the 3 symmetrically spaced screws and bolts **260**. Pairs of luer-lock fittings **290** were installed in tapped holes **295** on the circumference of the bottom and top. A fifth luer-lock fitting **210** was installed in a tapped hole **285** on the upper face of the top.

A closed-loop circuit for perfusion was formed by establishing two sets of connections using approximately 18"-long Tygon LFL L/S size #13 tubing (**300** and **305**, respectively) with male luer-lock×¼"-ID barbed 316 stainless steel fittings **315** on both ends. One length of tubing **300** connected one of the luer-lock fittings **210** on the bottom of the reservoir **220** to one of the luer-lock fittings **210** on the cell-seeded device **1**; this tubing functioned as a flow passage to introduce flowing medium **11** into the cell-seeded device. A second length of tubing **305** connected one of the luer-lock fittings **210** on the top of the reservoir to the remaining luer-lock fitting **210** on the cell-seeded device; this tubing functioned as a flow passage to remove medium from the cell-seeded device. A 316 stainless steel luer-lock plug **215** was installed on the remaining fitting on the bottom of the reservoir. This plug was removed when medium in the reservoir was sampled or the reservoir was drained.

A volume of perfusate **11**, typically ranging from 10–20 mL, then was added to the reservoir **220** through the luer-lock fitting **210** on the upper face of the top **240** of the reservoir. During this addition the unconnected luer-lock fittings **210** on the circumference of the top of the reservoir were unplugged to allow venting. Luer-lock plugs **215** then were installed on the remaining luer-lock fittings **210** prior to transfer of the assembled circuit from the biological safety cabinet to the incubator. In the incubator the tubing **300** was loaded into the head of a pump **230** (typically a peristaltic pump comprising a Cole-Parmer Easy-Load pump head with PSF housing and Cole-Parmer stainless steel rotor regulated by a Cole-Parmer controller). The device **1** was reconnected to the oxygenated fluid supply **4** as described in Example 1. The pump then was powered to the desired volumetric flow rate (ranging between 0 and 12 mL/min but typically 0.1–1.5 mL/min), and the liquid **11** in the reservoir recirculated through the tubing **300** and pump head, into the cell-seeded device **1**, through the tubing **305**, and back into the reservoir. During this procedure the device may be tilted to facilitate removal of air bubbles.

Two methods of further operation of the perfusion circuit were implemented. In one method (Perfusion Method I) the medium **11** was completely exchanged for fresh medium after every 24–72 hours of operation; in the second method (Perfusion Method II) the medium was never exchanged but rather was introduced initially as a large enough volume to support the cells for a period of one to many days. For Perfusion Method 1 the pump **230** first was stopped, the oxygenated fluid supply **4** disconnected, and the perfusion circuit transferred into a biological safety cabinet. The medium remaining in the reservoir **220** was drained by removing the luer-lock plug **215** from the luer-lock fitting **210** on the bottom **250** of the reservoir not connected to the tubing **300** and then tilting the reservoir. The circuit then was reconfigured temporarily by detaching the tubing **305** from the luer-lock fitting **210** on the top **240** of the reservoir. After removing the luer-lock plug **215** from the luer-lock fitting on the top face of the top of the reservoir, the reservoir was refilled with the volume of medium added initially and the pump restarted. The pump was operated in this open-loop configuration for a period of time equal to the ratio of the volume retained after draining the circuit (calculated as the difference between the volume added initially and the volume drained) to the volumetric flow rate. The pump then was stopped and the circuit reconfigured to the original closed-loop configuration prior to transfer back to the incubator, reconnection to the oxygenated fluid supply, and restarting of the pump.

For Perfusion Method II the pump **230** was stopped, the oxygenated fluid supply **4** disconnected, the tubing **300** unloaded from the pump, and the perfusion circuit without the pump returned to the biological safety cabinet without emptying the device of liquid. A 750 μL sample was removed with a sterile pipette from the port **210** for sampling on the reservoir **220** (after unplugging the luer-lock plug **215** for the port for venting). The perfusion circuit then was returned to the incubator, the appropriate section of tubing reinstalled into the pump head, pump turned on, and oxygenated fluid supply reconnected to the device.

Although the above perfusion circuit had a closed-loop configuration, we anticipate forms of the embodiment described in this Example 2 with an open-loop configuration in which the flow path is from a supply reservoir, through a pump, through the device, and into a collection reservoir. Assembly and operation of this open-loop circuit follows the general procedures described above.

The procedures and operations described above resulted in a device that was operated for in vitro studies in a manner simulating the use of a LAD for extracorporeal treatment of human and animal subjects experiencing compromised liver function.

Example 3

Hepatocellular Detoxification Activity Based on Conversion of Alkoxyresorufin to Resorufin

Cytochrome P450 (CYP450) isozymes are Phase I mixed-function monooxygenases which, along with complemen-

tary Phase II conjugative enzymes, catalyze the biotransformation of xenobiotics as well as endogenous lipophiles into soluble compounds more easily cleared by the renal system. As monooxygenases the function of CYP450 isozymes requires the presence of molecular oxygen. Accordingly, the activity of CYP450 isozymes is believed to depend on the availability of oxygen.

The O-dealkylation of non-fluorescent alkoxyresorufin ethers to fluorescent resorufin offers a tool for investigating the activities of CYP450 isozymes. For example, the dealkylation of the phenoxazone ethers benzyloxyresorufin (BROD) and ethoxyresorufin (EROD) has allowed researchers to study the activity of the individual isozymes CYP1B2 and 1A1, respectively. To measure CYP450 activity in static cultures, standard complete medium **11** with 1% NBCS was replaced in the cell-seeded device **1** from Example 1 with complete medium lacking serum but containing 5 μM of BROD or EROD (Molecular Probes, Eugene, Oreg.) and 80 μM of dicumarol (Sigma Chemical Co., St. Louis, Mo.). Dicumarol was included in the incubation to limit cytosolic degradation of resorufin, the product of the dealkylation of alkoxyresorufin by CYP450 isozymes, by subsequent Phase II reactions.

Samples were collected after incubation for 3 hours and analyzed in a Turner 450 fluorimeter at excitation and emission wavelengths of 540 nm and 585 nm, respectively. Data were collated as stimulation indices (ratios of fluorescence in samples after biotransformation for three hours to fluorescence in aliquots of equal volume prior to addition to the cell-seeded device **1**). Data were expressed as ratios of the stimulation index for a device containing a gas-permeable cell culture support being tested to the stimulation index for cells from the same donor and day post seeding but seeded into equivalently-sized 60 mm-diameter tissue-culture dishes. This assay typically was performed on the first day post seeding of cells but also was occasionally performed up to seven or more days post seeding.

In this assay, conversion of alkoxyresorufin to resorufin corresponds to an increase in fluorescence, such that the stimulation index and detoxification activity correspond to the level of P450 activity of a particular set of isozymes and intensity of the detoxification response of cultured hepatocytes.

Example 4

Activity of Hepatocellular P450 Isozymes Based on Clearance of Diazepam

Metabolism of the drug diazepam provided an alternative method to evaluate the activity of P450 isozymes in hepatocytes supported in device **1** of Example 1. The clearance of diazepam and its biotransformation to nordiazepam and temazepam were evaluated using a method similar to that of Jauregui et al. (H O Jauregui, S F Ng, K L Gann, D J WaxmanXenobiotic induction of P-450 PB-4 (IIB1) and P-450c (IA1) and associated monooxygenase activities in primary cultures of rat hepatocytes, Xenobiotica 21:1091–1106, 1991). In this method the medium added to the device from Example 1 on the first day post seeding after installation of the cap **110** was supplemented with 70 μM diazepam (Sigma). A 750 μL sample was collected after incubation for 24 hours and frozen until extraction; a sample of the diazepam-supplemented medium prior to addition to the device also was collected and frozen. Solid-phase extraction was performed with Oasis cartridges (Waters Corp., Milford, Mass.) and a Waters extraction manifold. Cartridges were primed sequentially with first methanol and then reverse-osmosis deionized water (RODI). Samples were loaded onto the column and washed with 5% methanol in RODI, eluted off the column with methanol, and evaporated and reconstituted with 250 μL of a mobile phase of 65% methanol/35% 0.01 M ammonium acetate at pH 6.0. Reverse-phase HPLC was conducted at a flow rate of 1.0 mL/min on a Waters Micro-Bondpak C18 column at 24.5° C. Elution was measured by optical absorbance at 254 nm. Diazepam eluted at approximately 11 minutes; the metabolites nordiazepam and temazepam eluted at 10 and 8 minutes, respectively. Data were expressed as percentage of the initial diazepam cleared and as percentages of the initial diazepam converted to nordiazepam or temazepam.

In this assay, high percentages of initial diazepam cleared and converted to nordiazepam and temazepam correspond to high P450 isozyme activity. The higher these percentages, the greater the P450 activity.

Example 5

Hepatocellular Detoxification Activity Based on Metabolism of Lidocaine

Metabolism of the drug lidocaine provided another method to evaluate the detoxification activity of hepatocytes supported in device **1** of Example 1. The clearance of lidocaine was evaluated using a method similar to that of Nyberg et al. (S L Nyberg, H J Mann, R Remmel, W-S Hu, F B Cerra, Pharmacokinetic analysis verifies P450 function during in vitro and in vivo application of a bioartificial liver, ASAIO J 39:M252–256, 1993). In this method the medium added to the device from Example 1 on the first day post seeding after capping was supplemented with 740 μM lidocaine (Paddock Laboratories Inc., Minneapolis, Minn.). A 600 μL sample was collected after incubation for 24 hours and frozen until extraction; a sample of the lidocaine-supplemented medium prior to addition to the device also was collected and frozen. Solid-phase extraction was performed with Oasis cartridges (Waters Corp.) and a Waters extraction manifold. Cartridges were primed 99% MeOH 1% HCl and 0.5 M Borax. Samples were loaded onto the column and washed with 0.5 M Borax, eluted with MeOH/HCl and then evaporated and reconstituted with 250 μl of mobile phase of 85% (50 mM NH_4HPO_4+10 mM hexanesulfonic acid, pH 3.0)/15% acetonitrile. Reverse-phase HPLC was conducted at a flow rate of 1 mL/min on a Microsorb C8 column (Rainin Instrument Co., Woburn, Mass.) at room temperature. Elution was measured by optical absorbance at 214 nm. Lidocaine eluted at approximately 37 minutes; MEGX, the major metabolite, eluted at 27 minutes. Data were expressed as percentage of the initial lidocaine cleared.

In this assay, high percentages of initial lidocaine cleared and converted to metabolites correspond to high P450 isozyme activity. The higher these percentages, the greater the P450 activity.

Example 6

Hepatocellular Ureagenesis of Medium Based on Synthesis of Urea

Urea synthesis was determined by either of two methods for device **1** of Examples 1 and 2.

Method 1

The clearance of ammonia, its salts, and aminated components in the medium, through deamination and ureagenesis, is believed to be a critical function of hepatocytes in vivo and a desired function of these cells as part of a LAD. Deamination results in the formation of urea, which in vivo is cleared by the renal system. The synthesis of urea was measured with devices **1** from Examples 1 and 2 using a colorimetric method for the determination of nitrogen, available as Kit #640-B from Sigma Diagnostics (St. Louis,

Mo.). Samples were collected periodically after seeding of cells into devices and treated with urease to hydrolyze urea to NH_3 and CO_2. The resulting NH_3 then was reacted with hypochlorite and phenol in the presence of the catalyst, sodium nitroprusside, to form indophenol. The optical absorbance of the resulting solution of indophenol was measured at 570 nm and converted to concentration of urea in the original sample using a standard curve. Data were expressed as amount of urea produced per device per day by multiplying the concentrations by volume of medium 11 in the device and dividing by number of days since sampling.

Method 2

In this assay, hepatocyte cultures were challenged with ammonium chloride and ornithine in order to increase urea production. The synthesis of urea is part of the process of deamination and ureagenesis. Accordingly, increases in the synthesis of urea, particularly in response to challenge with exogenous ammonia, represent increased functional capacity to deaminate media. After challenge, the medium from the cultures was tested to measure the amount of urea produced.

A stock solution of 1 M ammonium chloride (NH_4Cl; Sigma #A-0171) was prepared by adding 1.07 g NH_4Cl to 10 mL of RODI. The solution was stored at 4° C. until use. A stock solution of 0.2 M ornithine (Sigma #O-6503) was prepared by adding 0.34 g to 10 mL of RODI. This solution also was stored at 4° C. until use.

Culture plates were then rinsed with 1× phosphate buffered saline (PBS). To each plate was added 10 μL of each ammonium chloride and ornithine for each 1 mL of William's 1% medium (supplemented with the following: 4.5 g/L glucose, 0.5 U/mL bovine insulin, 7 ng/mL glucagon, 7.5 μg/mL hydrocortisone, 10 mM HEPES, 20 ng/mL, EGF, 20 mM glutamine, 10 IU penicillin, and 10 μg streptomycin and 1% new-born calf serum (NBCS)), that is, 40 μL were added as 4 mL of William's medium is used to culture porcine hepatocytes in 60 mm culture plates. Plates with regular culture medium not treated with ammonium chloride and ornithine were included as controls. The plates were returned to the incubator for 24 hours. After incubation, 2 mL of medium was removed from plates and transferred into appropriately labeled test tubes (Falcon #2054) and the tubes stored at −20° C.

The amount of urea present in frozen samples was determined using a Blood Urea Nitrogen (BUN) kit (Sigma, kit #535). Thawed 40 μL samples were added with 3.0 mL of the BUN acid reagent and 2.0 mL of the BUN color reagent to 100 mm disposable borosilicate glass tubes. Tubes were incubated at 100° C. for 10 minutes and then at room temperature for 3–5 minutes. Once cooled tubes were mixed well using the Vortex shaker. Resulting solutions were sampled colorimetrically in 96-well plates in triplicate at 546 nm. Optical absorbances were converted to concentrations of urea using a standard curve and data expressed as amount of urea produced per device per day by multiplying concentrations by volume of medium 11 in the device and dividing by number of days since sampling.

In this assay, high rates of formation of urea correspond to expected high levels of deamination and clearance of ammonia, clinically desired target functions. The higher the rate of urea synthesized, the greater the expected level of deamination.

Example 7

Synthesis and Secretion of Helpatocellular Proteins Based on Secretion of Albumin

The synthesis and secretion of serum proteins, including albumin, is believed to be a critical function of hepatocytes in vivo and in a LAD. Albumin is used as a marker of the secretory activity of hepatocytes. The secretion of albumin was measured using a standard competitive ELISA with samples for device 1 of Examples 1 and 2. Samples were collected periodically after seeding cells 40 into devices 1 and frozen until analyzed by ELISA. For ELISAs individual wells in 96-well plates were coated overnight with 200 μg/mL porcine albumin (Accurate Chemical, Westbury, N.Y.). Following a wash step with Tween 20 (Pierce, Rockford, Ill.), 50 μL of a sample or standard (Accurate) in individual wells of a multiwell plate was incubated with a horseradish peroxidase-conjugated goat anti-pig albumin antibody (Bethyl Labs, Montgomery, Tex.) for 90 minutes. Color was produced by addition of O-phenylenediamine dihydrochloride (Pierce) and the reaction stopped by adding H_2SO_4. The optical absorbance of individual wells was measured at 490 nm using a Molecular Devices SpectraMax 250 plate reader with SoftMax Pro software and converted to concentration of albumin in the original sample using a standard curve. Data were expressed as amount of albumin produced per device per day by multiplying the concentrations by volume of medium in the device and dividing by number of days since sampling.

In this assay, higher rates of albumin secretion are marks of clinically desired function by differentiated hepatocytes.

Example 8

Determination of Adherent Cell Mass Based on Measurement of Total Hepatocellular Protein

Hepatocyte cell mass and attachment was determined for device 1 of Examples 1 and 2 based on amount of associated protein using a BCA Protein Assay Kit (Pierce, catalogue no. 23225). Assay reagents were prepared according to the instructions contained therein. Reagent A was prepared as 1 L of base reagent with sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.2 N NaOH. Reagent B was prepared as a 25 mL of 4% copper sulfate solution. Bovine Serum Albumin (BSA, Pierce, Cat. No 23209) was used as standards at 2 mg/mL in 0.9% sodium chloride and 0.05% sodium azide.

Devices 1 were removed from the incubator, caps 110 removed, and medium 11 aspirated from cultures. The medium was replaced with phosphate buffered saline (PBS) and cultures frozen at 0° C. for 24 hours. Cultures were then thawed and scraped from gas-permeable, liquid-impermeable membranes 30 into appropriately-labeled test tubes.

A solution of working reagent was prepared by combining 50 parts reagent A with 1 part reagent B. A set of protein standards was prepared by serially diluting 0 to 2 mg/mL of the stock BSA solution in the same diluent. Next, 10 μL of each sample or standard and 200 μL of the working reagent were sequentially was added to individual wells of 96-well plates. Samples were mixed for 30 seconds by gently shaking prior to covering and incubating at 37° C. for 30 minutes. Wells then were assessed colorimetrically at 560 nm. Protein concentrations for unknowns were determined from a standard curve.

Example 9

Comparison of Alternative Gas-Permeable Membranes for Hepatocytes in Cell Culturing Devices

The apparatus of Example 1 was used to evaluate the potential of different gas-permeable, liquid-impermeable membranes as supports for hepatocytes in culture. Hepatocytes lose their functionality if not seeded onto a solid support following isolation from a donor. Further, the level of function that they achieve in culture can depend critically

on the properties of the culture support, including whether it is coated with a layer or gel of collagen or substances that promote adhesion and function.

Membranes evaluated included Polyflex® (a non-porous 0.002"-thick film of polystyrene manufactured by Plastics Suppliers, Inc., Columbus, Ohio), Breathe-Easy™ (a non-porous film of polyurethane manufactured by Diversified Biotech, Boston, Mass.), a three-layered co-extruded film of styrene-butadiene-styrene/ethyl vinyl acetate/styrene-butadiene-styrene (SBS/EVA/SBS, a non-porous film supplied by BASF, Germany to Baxter Healthcare Corporation, Nivelles, Belgium), a two-layered co-extruded film of styrene-butadiene-styrene/polyethylene (SBS/PE, manufactured by Cypress Cryovac/Sealed Air Corp., Rochester, N.Y.), non-porous polyester sheet (manufactured by Perfecseal Inc., Philadelphia, Pa.), HDPE in the form of TYVEK® 1073 (a microporous non-woven membrane composed of USP Class VI polyolefin and manufactured by E. I. du Pont de Nemours and Co, Inc., Wilmington, Del.), microporous polyethylene (PE) sheet (obtained from 3M, Inc. (Minneapolis, Minn.) with a mean pore size of 1.7 μm as determined by a bubblepoint test), PolySep® (microporous polypropylene sheet manufactured by Micron Separations, Westborough, Mass.), microporous poly (tetrafluoroethylene) (PTFE) and Hydrulon® (microporous hydrophobized nylon 6,6) (both manufactured by Pall Corporation, Port Washington, N.Y.), and track-etched polycarbonate (manufactured by Micron Separations). Membranes with two different pore sizes were examined for the microporous PTFE, Hydrulon, and track-etched polycarbonate.

Each of the membranes tested would be expected to have sufficient permeability to oxygen and carbon dioxide to support direct transmembrane oxygenation of adherent hepatocytes. Further, each of these membranes would be expected to be water-impermeable for the pressure differences across the membrane expected for oxygenation and perfusion in a cell culture device or organ assist system.

Each of these membranes was evaluated without coating with collagen type I prior to seeding of cells at a density of 2×10^6 hepatocytes per device. An oxygenated fluid supply with 19% O_2, 10% CO_2, and balance N_2 was used. Data for detoxification activity by methods of conversion of alkoxyresorufin to resorufin (Example 3) for cells from donor 54 (polyurethane, PTFE, and hydrophobized nylon), donor 61 (HDPE), donor 76 (track-etched polycarbonate), donor 80 (SBS/EVA/SBS, PS/PE, and PE), donor 84 (polyester), and donor 89 (polystyrene) on day 1 post seeding are presented in Table 1.

TABLE 1

Detoxification Activity for Hepatocytes in Static Cultures in Cell Culturing Devices for Different Gas-Permeable, Liquid-Impermeable Membranes

Gas-Permeable Membrane		Detoxification Activity	
Material (Source)	Pore Size	BROD	EROD
Polystyrene (Polyflex ®, Plastics Suppliers)	Nonporous	1.44	1.11
Polyurethane (Breathe-Easy ™, Diversified Biotech)	Nonporous	0.33	0.84
SBS/EVA/SBS (BASF)	Nonporous	---	0.65
Co-extruded poly-ethylene/polystyrene (Cryovac)	Nonporous	—	0.72
Polyester (Perfecseal)	Nonporous	1.10	1.68

TABLE 1-continued

Detoxification Activity for Hepatocytes in Static Cultures in Cell Culturing Devices for Different Gas-Permeable, Liquid-Impermeable Membranes

Gas-Permeable Membrane		Detoxification Activity	
Material (Source)	Pore Size	BROD	EROD
HDPE (TYVEK ® 1073, Dupont)	4.2 μm	0.22	0.81
Polyethylene (3M)	<10 μm	0.77	0.95
Polypropylene (Polysep ®, Micron Separations)	0.45 μm	0.48	0.74
PTFE (Pall)	0.1 μm	0.38	0.87
PTFE (Pall)	1 μm	0.47	0.93
Hydrophobized nylon 6,6 (Hydrulon ®, Pall)	0.2 μm	0.35	1.38
Hydrophobized nylon 6,6 (Hydrulon ®, Pall)	1.2 μm	0.31	1.29
Track-etched polycarbonate (Micron Separations)	0.1 μm	0.45	0.91
Track-etched polycarbonate (Micron Separations)	1 μm	0.24	0.48

Highest levels of detoxification activity for the biotransformation of BROD to resorufin were observed for cells cultured in devices comprising Polyflex® or the polyester membrane. The microporous polyethylene membrane supported intermediate levels of detoxification activity for conversion of BROD in the cell-seeded device 1. Highest levels of detoxification activity for the biotransformation of EROD to resorufin were observed for cells cultured in devices comprising Polyflex®, the polyester membrane, or Hydrulon®. Increasing pore size decreased detoxification activity for both alkoxyresorufins on track-etched polycarbonate but had relatively minor effects for microporous PTFE and hydrophobized nylon 6,6. In general, detoxification activity was more dependent on membrane type for BROD than for EROD. Further, the highest overall detoxification activities were observed for cell-seeded devices comprising nonporous polystyrene or polyester.

These results demonstrate how changing the chemical composition and physical structure of a gas-permeable, liquid-impermeable membrane 30 can affect the ability of hepatocytes 40 cultured in a device 1 to perform detoxification.

The apparatus of Example 1 also was used to compare the ureagenesis potential of hepatocytes cultured in a device 1 comprising Polyflex® with hepatocytes 40 cultured in equivalently sized 60 mm-diameter tissue-culture dishes. Tissue-culture dishes present to cells a surface effectively impermeable to gas, including oxygen, relative to Polyflex®: Polyflex® has a permeability to oxygen on the order of 2.0×10^4 mL/m^2-day-atm, whereas the permeability to oxygen of tissue-culture dishes is below the ability of state-of-the-art techniques to measure.

For this study 2×10^6 hepatocytes from donor 93 were seeded into either a device 1 with Polyflex® or into a tissue-culture dish. The device was supplied with a gas feed of 19% O_2, 10% CO_2, and balance N_2. Data for the synthesis of urea, in terms of both the basal level (open symbols based on Example 6, Method 2 without addition of exogeneous ammonia and ornithine) and in response to challenge with 20 mM exogenous ammonia (closed symbols based on Example 6, Method 2 with addition of exogeneous ammonia and ornithine), is presented in FIG. 11 for devices (squares) and tissue-culture dishes (circles). The basal level of synthesis of urea for devices over the course of 11 days of culture is indistinguishable from the basal level for hepato-

cytes maintained in tissue-culture dishes over this period of time. Further, the synthesis of urea in response to challenge with ammonia is the same as or greater in the devices than in the tissue-culture dishes. These results demonstrate that not only can higher levels of detoxification be achieved upon culturing hepatocytes on a gas-permeable membrane such as Polyflex® but also ureagenesis, particularly in response to exogenous ammonia, can be enhanced by culturing hepatocytes adherent on a gas-permeable membrane in a device.

Example 10

Effect of Surface Treatment of Gas-Permeable Membranes on Hepatocytes in Cell Culturing Devices

The effect of modification of the surface of gas-permeable, liquid-impermeable membranes **30** also was evaluated using the apparatus of Example 1. Specific modification (e.g., by coating or covalent attachment of specific molecules) and non-specific modification (e.g., by treating the surface with an oxidizing chemical or physical process) alters the cell-adhesive properties of surfaces and the ability of these surfaces to promote cell function. These treatments modify only the surface of the cell-supporting membrane and not the membrane's bulk properties.

We evaluated the effects of two independent treatments **41**, applied prior to seeding cells **40**, to control the function of hepatocytes cultured on gas-permeable, liquid-impermeable membranes **30** in a device **1**. One treatment comprised coating the surface of the membrane with a sterile solution of 1.1 mg/mL collagen type I for 45 minutes, followed by rinsing with sterile phosphate-buffered saline. The resulting coating was on the order of one to a few molecules of collagen thick. This type of treatment enhances the adhesion of many types of cells to many otherwise less-adhesive surfaces. The second treatment comprised exposure of the cell-supporting side of membranes to a corona discharge to oxidize this surface. Treatment of polystyrene dishes with a corona discharge oxidizes the cell-supporting surface, such that the dyne level (critical surface tension) is 40–50 dynes/cm, and promotes cell attachment for many types of cells. This treatment modifies only a thin surface-most layer of the surface, of thickness less than approximately 1 micron, without increasing the thickness of the membrane or depositing macroscopic amounts of material onto the membrane. This treatment was applied to both the non-porous Polyflex® (polystyrene) and porous TYVEK® 1073 (non-woven HDPE) membranes such that their dyne levels (critical surface tensions) became approximately 45 dyne/cm.

Table 2 presents data for detoxification activity by methods of conversion of alkoxyresorufin to resorufin (Example 3) for cells, seeded at 2×10^6 hepatocytes per device **1**, from donor 61 (HDPE), donor 76 (track-etched polycarbonate), donor 80 (SBS/EVA/SBS, PS/PE, and PE), and donor 89 (polystyrene) on day 1 post seeding for devices supplied with 19% O_2, 10% CO_2, and balance N_2. Changes in detoxification in response to surface treatment **41** depended on the specificity of detoxification (BROD or EROD) as well as membrane type. For some membranes (e.g., HDPE and track-etched polycarbonate with 1 micron-diameter pores), coating with collagen improved both specificities of detoxification. For other membranes (e.g., polystyrene, co-extruded polyethylene/polystyrene, track-etched polycarbonate with 0.1 micron-diameter pores) coating with collagen improved only one specificity of detoxification with minor changes in the alternative specificity. The remaining membranes experienced minor reductions in detoxification upon treatment with collagen.

TABLE 2

Effect of Surface Treatment of Gas-Permeable Membranes on Detoxification Activity for Hepatocytes in Static Cultures in Cell Culturing Devices

Gas-Permeable Membrane	Surface Treatment		Resorufin Activity	
Material	Collagen coated	Corona treated	BROD	EROD
Polystyrene	No	No	1.44	1.11
	Yes	No	2.04	0.94
	No	Yes	1.64	1.08
	Yes	Yes	1.51	1.60
SBS/EVA/SBS	No	No	—	0.65
	Yes	No	—	0.45
Co-extruded polyethylene/polystyrene	No	No	—	0.72
	Yes	No	—	0.79
HDPE	No	No	0.22	0.81
	Yes	No	0.32	0.99
	No	Yes	0.60	1.00
Polyethylene	No	No	0.77	0.95
	Yes	No	0.54	0.86
Polycarbonate (0.1 μm pores)	No	No	0.45	0.91
	Yes	No	0.33	1.23
Polycarbonate (1 μm pores)	No	No	0.24	0.48
	Yes	No	0.59	0.91

Surface treatment with corona discharge in the absence of coating with collagen significantly improved the detoxification activity of both polystyrene and HDPE. Sequential treatment by corona discharge followed by coating with collagen further improved the overall detoxification activity of polystyrene. This combination, when applied to Polyflex®, produced the system with the greater detoxification activity.

Culturing hepatocytes in a device **1** comprising corona discharge-treated, collagen-coated Polyflex® also supported sustained detoxification activity over the evolution of a culture. For these evaluations detoxification activity based on the biotransformation of BROD and EROD (Example 3) was measured on days one, three, and seven post seeding. The values for BROD decreased slightly (from 1.51 on day one to 1.47 on day three to 1.20 on day seven), whereas the values for EROD increased from day one to day three (1.60 to 4.25) before decreasing on day seven (0.87). All of these values for detoxification activity were higher than for other membranes.

These results demonstrate the utility of modifying gas-permeable, liquid-impermeable membranes **30** with one or more surface treatments **41**, in which only the surface of the membrane exposed to cells **40** (and not the membrane's gas permeability) is modified, in order to achieve desired increases in hepatocyte function in a cell culturing device **1**.

Example 11

Effect of Oxygenation on Hepatocytes in Unperfused Cell Culturing Devices

The apparatus of Example 1 was used to compare the effect of various gas compositions on the functions of static hepatocyte cultures. The partial pressure of oxygen to which cells, and in particular hepatocytes, are exposed can significantly influence their function. Direct transmembrane oxygenation of adherent cells can be achieved in device **1** by feeding a gas with desired concentration of oxygen to one side of a gas-permeable membrane and culturing cells on the opposing side. This mode of oxygenation offers reduced resistance to the transport of oxygen to cells compared to

oxygenation through saturation of medium with oxygen. Further, direct transmembrane oxygenation effectively decouples oxygenation and perfusion of cells.

For this Example oxygenation of adherent hepatocytes in static cultures was controlled by setting the concentration of oxygen in the gas fed to the device **1** and contacted with the gas-permeable, liquid-impermeable cell-supporting membrane **30**. Two different membranes were examined: corona discharge-treated, collagen-coated polystyrene (Polyflex®) and uncoated, non-woven HDPE (TYVEK® 1073). Gas compositions tested comprised 10% CO_2 with 0%, 19%, 40%, 60%, 65%, or 90% O_2, and balance N_2. Carbon dioxide was included to maintain a physiological pH based on the used of a standard bicarbonate-based buffer.

FIGS. **12***a–d* show data for the attachment of cells one day post seeding (Example 6), for ureagenesis (Example 5), for detoxification activity based on biotransformation of alkoxyresorufins (Example 3), and detoxification activity based on the metabolism of lidocaine (Example 5) for hepatocytes 40 cultured statically in devices **1** containing 0.002"-thick corona-treated, collagen-coated polystyrene. Data were obtained for 2×10^6 hepatocytes seeded from donor 100 and 101 and were expressed relative to devices fed 19% O_2. Attachment one day post seeding (FIG. **12***a*), measured as the amount of total protein associated with adherent cells, doubled with an increase in concentration of O_2 in the feed gas to 60%.

Data for the synthesis of urea, in terms of both the basal level (open symbols) and in response to challenge with 20 mM exogenous ammonia (closed symbols), are presented in FIG. **12***b* for devices **1** on days two (squares) and five (circles) post seeding. Basal levels are relatively independent of the concentration of O_2 in the feed gas. Conversely, challenged rates of urea synthesis increased with concentration of O_2 on day two post seeding but decreased with concentration of O_2 on day five post seeding.

Data for detoxification activity based on the conversion of BROD (squares) and EROD (circles) to resorufin is presented in FIG. **12***c* for days one (closed symbols) and four (open symbols) post seeding. On day one post seeding greatest detoxification activities were observed for an intermediate concentration of O_2 of 40%. On day four post seeding detoxification activity for BROD was independent of concentration of O_2, but detoxification activity for EROD decreased monotonically with concentration of O_2 on this day in culture.

Complementary trends were observed for the amounts of metabolites of lidocaine (FIG. **12***d*). On day two post seeding (closed squares) the amount of metabolites increased slightly with increasing concentration of O_2, but on day five post seeding (closed circles) the amount of metabolites for lidocaine decreased sharply with increasing concentration of O_2.

These results demonstrate that controlling the concentration of oxygen fed into a device **1** comprising corona discharge-treated, collagen-coated Polyflex®, thereby controlling the extent of oxygenation of static cultures of hepatocytes 40, can be used to control a wide range of functions of hepatocytes. Further, by selecting the concentration of oxygen and varying it over different days in culture, different functions can either be promoted or downregulated.

Similar types of data were obtained for hepatocytes 40 cultured statically in devices **1** comprising TYVEK® 1073 as the gas-permeable, liquid-impermeable membrane/cell support **30**. Hepatocytes in this system were seeded at a density of 2×10^6 cells per device and evaluated for the effect of oxygenation on their detoxification activity by methods of conversion of alkoxyresorufin to resorufin (Example 3) on day 1 post seeding for cells from donor 73 and clearance of diazepam (Example 4) on days 2 and 4 post seeding for cells from donor 78, ureagenesis based on basal levels of synthesis of urea (Example 5) for day 3 post seeding for cells from donor 76, and synthesis and secretion of albumin (Example 6) for cells from donor 74. Data were collected for these assays from different porcine donor and are summarized in FIG. **13** and Tables 3 and 4.

FIG. **13** shows that greatest detoxification activities for BROD (closed squares) and EROD (open squares) were observed for transmembrane oxygenation with 65% O_2. Exposure to higher or lower concentrations of O_2 resulted in lower detoxification activity: exposure to 0% O_2 asphyxiated cells, exposure to 19% and 40% O_2 was limiting for detoxification activity, and exposure to 90% O_2 was hyperoxic and deleterious for cells. Similarly, Table 3 shows that higher percentages of diazepam were cleared and metabolized to nordiazepam and temazepam for an increase in oxygenation to 40% from 19%.

TABLE 3

Metabolism of Diazepam for Hepatocytes in Static Cultures in Cell Culturing Devices as Function of Oxygenation

	%-Diazepam Cleared	%-Diazepam Converted to Nordiazepam	%-Diazepam Converted to Temazepam
Day 2			
19% O_2	47.8%	3.0%	2.9%
40% O_2	51.6%	3.3%	3.2%
Day 4			
19% O_2	39.7%	0.8%	1.5%
40% O_2	52.3%	1.8%	3.7%

Further, devices **1** comprising TYVEK® 1073 and connected to 19% and 65% O_2 produced 20.5 and 42.5 μg/day of urea, respectively, demonstrating that the exposure to a more oxygen-rich gas increased the level of ureagenesis on this membrane material.

As shown in Table 4, a different trend with respect to level of oxygenation was observed for albumin secretion on TYVEK® 1073 in devices **1**: the maximum rate of secretion of albumin was detected for exposure to 19% O_2, with lower rates for exposure to higher concentrations of O_2. Together these results show the potential benefits of direct transmembrane oxygenation, controlled by manipulating the concentration of O_2 in the gas, on control of hepatocyte function, and how change of gas composition can be used to manipulate hepatocyte function.

TABLE 4

Secretion of Albumin for Hepatocytes in Static Cultures in Cell Culturing Devices as Function of Oxygenation

	Rate of Albumin Secretion (μg/day)			
% O_2 in Device Feed Gas	Day 3 Post Seeding	Day 6 Post Seeding	Day 8 Post Seeding	Day 10 Post Seeding
19	0.186	0.342	2.699	3.443
40	0.076	0.075	0.227	0.254
65	0.063	0.052	0.082	0.082
90	0.055	0.023	2.98	0.567

Example 12

Effect of Perfusion on Hepatocytes in Cell Culturing Devices

The apparatus of Example 2 was used to compare the effect of perfusing hepatocyte cultures at different volumetric flow rates in laboratory-scale devices comprising corona discharge-treated, collagen-coated Polyflex®. Devices **1** were seeded with 2×10^6 hepatocytes from donor 96, loaded with 10 mL of complete medium as perfusate, and fed gas containing 19% O_2, 10% CO_2, and balance N_2. FIG. **14** presents data for both basal levels of synthesis of urea (open symbols) and rates of synthesis of urea in response to challenge with 20 mM exogenous ammonia (closed symbols) for devices perfused at 1.5 mL/min (squares) and at 0.1 mL/min (circles). Data are expressed as percentages of static controls. Relative increases in the synthesis of urea, expected to be reflective of greater deamination, were observed for nearly every day in culture examined for both volumetric flow rates and for both basal and ammonia-challenged levels of urea. Neither volumetric flow rate was preferred for both greater basal and challenged ureagenesis. Data for devices seeded with 4×10^6 hepatocytes from donor 108 and 109, operated under similar conditions but with higher volumetric flow rates up to 12 mL/min, similarly showed no effect of flow rate on ureagenesis. These results demonstrate that devices **1** can be operated successfully with a range of volumetric flow rates without deleterious effects of hepatocyte function.

The apparatus of Example 2 also was used to compare the effect of perfusing hepatocyte cultures at a constant volumetric flow rate—but with different volumes of perfusate on cell growth and ureagenesis in devices comprising corona discharge-treated, collagen-coated Polyflex®.

For studies of cell growth and ureagenesis devices **1** were seeded with 2×10^6 hepatocytes from donor 96, loaded into perfusion circuits with 10 mL of complete medium as perfusate, and fed gas containing 19% O_2, 10% CO_2, and balance N_2. Table 5 presents data for the growth of cells, measured as cell mass by determination of total protein associated with adherent hepatocytes, for covered and capped devices. Data for cell density is expressed as percentage of cell mass observed for cultures in tissue-culture dishes. The static covered device and capped device perfused at 0.1 mL/min show increases in cell mass relative to tissue-culture dishes on day three post seeding. All configurations show extensive increases in cell mass by day nine post seeding. These results demonstrate that perfusion is not deleterious to growth of cultures.

TABLE 5

Effect of Device Configuration and Perfusion on Growth of Hepatocytes in Cell Culturing Devices

	System		Cell Density (%-TC Control)	
Configuration	Volume (mL)	Perfusion Rate (mL/min)	Day 3	Day 9
Device 1 with Cover 100	4	Static	189	706
Device 1 with Cap 110	3	Static	79	774
Device 1 with Cap 110	10	0.1	127	933
Device 1 with Cap 110	10	1.5	91	706

FIG. **15** presents data for basal levels of synthesis of urea for single devices perfused at 1.5 mL/min with 10 mL (squares) or 20 mL (circles) of perfusate. Data again are expressed as percentages of static controls. Relative increases in the basal synthesis of urea, expected to be reflective of greater deamination, were observed for every day in culture examined for all three systems. Larger relative increases were observed for devices perfused with 20 mL than with 10 mL. These results demonstrate that controlling the volume of perfusate can be an effective means to control hepatocyte function, and in particular ureagenesis.

The apparatus of Example 2 further was used to compare the effect of perfusing hepatocytes cultures at 1.5 mL/min with different volumes of perfusate in devices **1** comprising TYVEK® 1073 as the gas-permeable, liquid-impermeable membrane/cell culture support **30**. In these studies devices were seeded with 2×10^6 hepatocytes, loaded with either 10 or 20 mL of complete medium as perfusate, and fed gas containing 40% O_2, 10% CO_2, and balance N_2. Hepatocytes were evaluated for the synthesis of urea (Example 6, Method 1) and synthesis and secretion of albumin (Example 7) for cells from donor 75 for days 1–13 post seeding. Data are presented in FIGS. **16***a* and **16***b*.

For almost all time points examined, the synthesis of urea and secretion of albumin was greater with 20 mL of perfusate than with 10 mL of perfusate. These results demonstrate that greater levels of ureagenesis and secretion of albumin can be obtained using perfusion and by increasing the volume of perfusate. This example shows the utility of perfusion with direct transmembrane oxygenation for improved function.

Example 13

Effect of Cell Seeding Density on Hepatocytes in Perfused Cell Culturing Devices

The economic efficiency, practicality, and utility of a cell culturing device can be influenced by the density of cells that can be supported in the device: support of greater densities of cells frequently is associated with lower costs and the potential to build smaller devices and units. It further is typically desired that increases in cell density do not result in a significant loss of function on a per cell basis.

To address these issues the apparatus of Example 2 also was used to compare the effect of perfusing hepatocyte cultures seeded at different initial densities of cells on ureagenesis in devices comprising corona discharge-treated, collagen-coated Polyflex®. Devices **1** were seeded with either 4×10^6, 8×10^6, or 12×10^6 hepatocytes **40** from donor 109, loaded into perfusion circuits with 10 μL of complete medium as perfusate **11**, fed gas **4** containing 19% O_2, 10% CO_2, and balance N_2, and perfused from Day 1 to Day 2 of culture at 1.5 mL/min. Ureagenesis was determined based on Method 2 of Example 6. Table 6 shows that the rate of synthesis of urea increased approximately linearly with initial seeding density: the rate of ureagenesis per cell seeded varied from 98 to 106 to 85 upon increasing the number of cells seeded per device from 4×10^6 to 8×10^6 to 12×10^6. These results demonstrate that the invention allows linear scaling of hepatocellular function with cell number.

TABLE 6

Ureagenesis for Hepatocytes in Perfused Cell Culturing Devices as Function of Cell Seeding Density

	Ureagenesis	
Cells Seeded Per Device	Total (μg/dev/day)	Per Cell Seeded (pg/cell seeded/day)
4×10^6	390	98
8×10^6	845	106
12×10^6	1019	85

The possible utility of manipulating oxygenation on the function of higher density, perfused cultures also was examined using the apparatus of Example 2. Devices **1** comprising corona discharge-treated, collagen-coated Polyflex® were seeded with either 4×10^6, 8×10^6, or 12×10^6 hepatocytes **40** from donor 105, **109**, and 110, loaded into perfusion circuits with 10 mL of complete medium as perfusate **11**, fed gas **4** containing 10% CO_2 with between **0** and 90% O_2 (and balance N_2), and perfused from Day 1 to Day 2 of culture at 1.5 mL/min. Ureagenesis again was determined based on Method 2 of Example 6. FIG. **17** depicts data for these studies plotted as percentage of 19% O_2 controls. For lower density cultures (e.g., closed squares for 4×10^6 cells seeded initially), the synthesis of urea in response to challenge with 20 mM exogenous ammonia was greatest under conditions of reduction of oxygenation compared to 19% O_2. Further, for this lower density increasing oxygenation decreased ureagenesis. In contrast, for higher density cultures (e.g., closed diamonds for 8×10^6 cells and closed triangles for 12×10^6 cells) the rate of ureagenesis was greatest for an intermediate extent of oxygenation with 60% O_2.

These results demonstrate that manipulation of oxygenation can be used as a further means of controlling the performance and function of hepatocytes supported in the new cell culturing devices and that the optimal level of oxygenation can depend on the density of cells supported.

Example 14

Scaling of Performance with Size of Cell Culturing Device

The economic efficiency, practicality, and utility of a cell culturing device further can be influenced by the ability to increase the overall size of the device without loss of per-unit-area function. For example, it typically is desired to have 10 times the total function in a device **1** seeded with **10** times as many cells but also **10** times the projected area of the gas-permeable, liquid-impermeable membrane **30**, such that the seeding density of cells does not change. This need is dictated by the inability to increase the density of cells seeded without bound without loss of function on a per cell basis.

The present invention allows at least two methods for increasing device size without losing function on a per-area basis: increasing the size of a single unit or coupling multiple units together in parallel, series, or both. The first method involves using devices with units that are larger in absolute size; the second method involves using modular units.

The feasibility of scaling absolute device size with single units was examined by comparing the performance of the apparatus of Example 2 with a larger version of the device **1** using the perfusion circuit of Example 2. This larger device is depicted schematically in FIG. **18** and was sized with five times the projected area of gas-permeable, liquid-impermeable membrane **30** as the device of Example 1. This sizing allowed this device to be applied to treat rats with liver failure; hence it was denoted the "rat-scale" device to distinguish it from the "laboratory-scale" or "lab-scale" device depicted in FIG. **4**.

The rat-scale device comprised a set of aluminum parts, including upper **60** and lower **70** housings, a frame **35**, and cap **110**, in addition to gas-permeable, liquid-impermeable membrane **30**, three silicone gaskets (Specialty Silicone Products, Inc., Balston Spa, N.Y.) **85**, **80**, and **90**, and ancillary screws and fittings **120**, **180**, **181**, and **210**. A membrane/frame assembly **400** was constructed and sterilized by gamma irradiation as described in detail in Example 1. The rat-scale device **1** was also assembled and handled (including seeding of cells **40**) as described in detail in Example 1 for the lab-scale device with the following exception: the rat-scale device was seeded. with cells suspended in 33 mL of medium rather than 4 mL of medium. The numbers of cells in the suspension was adjusted so that the seeding densities (per unit area) were similar for the two scales.

For this study ureagenesis in the lab- and rat-scale devices were compared for hepatocyte cultures obtained from donor **101** perfused with **20** mL of complete medium **11** at similar hydrodynamic shear stresses for Day 1 to Day 2 post seeding. Both devices were fed gas **4** containing 19% O_2, 10% CO_2, and balance N_2. Ureagenesis was determined based on Method 2 of Example 6. Table 7 shows that by adjusting the number of cells seeded in each device, similar densities of cells were obtained. Further, the rate of synthesis of urea was approximately five times greater in the rat-scale device (with approximately five times as many cells) compared to the lab-scale device, such that the rate of ureagenesis per unit area was similar in the two devices.

TABLE 7

Scaling of Ureagenesis with Size of Cell Culturing Device

		Cells Seeded		Ureagenesis	
Device Scale	Area (cm^2)	Total	Density ($cells/cm^2$)	(μg/day)	($\mu g/cm^2$ - day)
Lab	23	2.0×10^6	8.7×10^4	87	3.8
Rat	100	9.6×10^7	9.3×10^4	450	4.3

These results demonstrate that the invention allows linear scaling of hepatocellular function with size of the gas-permeable, liquid-impermeable membrane **30**.

The feasibility of scaling device size with a plurality of modular units was examined by comparing the performance of the apparatus of Example 2 with a modular system similar to this apparatus. FIG. **19** illustrates the configuration of such a modular system in which two devices **1** are placed in parallel in a single perfusion circuit. Pumps **230** drive both devices at the same volumetric flow rate from a single reservoir **220**, such that both devices share the same volume of perfusate 11. Both devices are also fed the same gas **21** from the same source **4**. With such a system the number of cells used to treat a perfusate can be increased without changing the density of cells in the system or the size of any individual unit.

For this study devices **1** were seeded with 8×10^6 hepatocytes **40** from donor 110, loaded as either single devices or paired parallel devices into perfusion circuits with 20 mL of complete medium as perfusate 11, and fed gas **4** containing 19% O_2, 10% CO_2, and balance N_2. Perfusion was conducted such that each device—whether as a singleton or paired in parallel—received 1.5 mL/min of complete

medium with 20 mM exogenous ammonia from Day 1 to Day 2 at 1.5 mL/min. Ureagenesis was determined based on Method 2 of Example 6. Table 8 shows that doubling the number of cells seeded into the system with a pair of devices doubles the rate of ureagenesis for the system, such that the rate of ureagenesis per unit is constant. These results demonstrate that the overall function of the system scales linearly with the number of modular units in the system, such that increasing the size of the system through addition of devices operated in parallel increases the overall performance of the system.

TABLE 8

Scaling of Ureagenesis with Modularity of Cell Culturing Device

Number Of Units	Total Area (cm^2)	Cells Seeded: Total	Cells Seeded: Density (cells/cm^2)	Ureagenesis (μg/day)	Ureagenesis (μg/unit/day)
Single	23	8.0×10^6	3.5×10^5	9.5×10^2	9.5×10^2
Pair in parallel	46	1.6×10^7	3.5×10^5	2.1×10^3	1.0×10^3

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method for culturing hepatocytes, the method comprising:

providing a gas-permeable, liquid-impermeable membrane having a first surface and a second surface;

seeding hepatocytes on the first surface of the gas-permeable, liquid-impermeable membrane, wherein the hepatocytes are seeded directly on the membrane or directly on a molecularly-thick coating material on the surface of the membrane;

contacting the hepatocytes directly with a nutrient-containing culture medium;

providing an oxygenated fluid to the second surface of the gas-permeable, liquid-impermeable membrane at a pressure sufficient to provide transmembrane oxygenation to the hepatocytes seeded on the first surface; and

culturing the hepatocytes under conditions that promote viability and function of the hepatocytes.

2. The method of claim 1, wherein the oxygen contained in the oxygenated fluid is at or above the critical partial pressure of oxygen.

3. The method of claim 1, wherein the hepatocytes are cultured in a device seeded with 2 to 20 billion hepatocytes.

4. The method of claim 1, wherein the hepatocytes are porcine, equine, ovine, bovine, rabbit, rat, canine, feline, or murine hepatocytes.

5. The method of claim 1, wherein the hepatocytes are human hepatocytes.

6. The method of claim 1, wherein the seeded hepatocytes are preserved hepatocytes.

7. The method of claim 6, wherein the preserved hepatocytes are cryopreserved, hypothermically stored, or lyophilized hepatocytes.

8. The method of claim 1, wherein the gas-permeable, liquid-impermeable membrane material is selected from the group consisting of polystyrene, polyolefin, polyethylene, polypropylene, polyvinylidene fluoride, polycarbonate, hydrophobic-treated nylon, polyurethane, polyester, layered styrene-butadiene-styrene/ethyl vinyl acetate/styrene-butadiene-styrene, and layered styrene-butadiene-styrene/polyethylene.

9. The method of claim 1, wherein the first surface of the gas-permeable, liquid-impermeable membrane is corona treated.

10. The method of claim 1, wherein the first surface of the gas-permeable, liquid-impermeable membrane is coated with a molecularly-thick coating of collagen.

11. The method of claim 1, wherein the concentration of oxygen in the oxygenated fluid is between about 0% to about 90% at a pressure of 2–5 psi.

12. The method of claim 11, wherein the concentration of oxygen in the oxygenated fluid is between about 19% to about 60% at a pressure of 2–5 psi.

13. The method of claim 11, wherein the concentration of oxygen in the oxygenated fluid is between about 40% to about 60% at a pressure of 2–5 psi.

14. The method of claim 1, wherein the concentration of oxygen in the oxygenated fluid is controlled to promote or downregulate cell function.

15. The method of claim 1, wherein the nutrient-containing culture medium is perfused.

16. The method of claim 1, wherein the nutrient-containing culture medium comprises blood plasma.

17. The method of claim 1, wherein the hepatocytes are seeded across the entire membrane from above the membrane.

18. The method of claim 1, wherein the hepatocytes are seeded directly onto the gas-permeable, liquid-impermeable membrane.

* * * * *

EXHIBIT E

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[54] STEM CELL IMMOBILIZATION

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[58] Field of Search 435/172.1, 374, 435/378, 397, 402, 405, 408, 455, 307.1, 308.1; 604/408

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[57] ABSTRACT

Haematopoietic stem cells (which term includes early progenitor cells) are immobilized on a substrate coated with a fibrin matrix and including a substance capable of both binding to the fibrin matrix and also having an RGD amino acid sequence for binding to the stem cells. The substance may be fibronectin or thrombospondin. The substrate is generally in the form of a closed bag formed of a carbon dioxide-permeable and oxygen-permeable plastics material which allows culturing of the stem cells. The cultured stem cells may re-engraft a patient following chemotherapy or to correct haemotological deficiencies. Stem cells may be harvested from peripheral blood onto the coated substrate. The stem cells in contact with the coated substrate are good candidates for gene therapy to introduce a heterologous gene e.g. employing a transfection vector.

18 Claims, No Drawings

STEM CELL IMMOBILIZATION

TECHNICAL FIELD

The present invention relates to a system for selectively immobilizing stem cells, for example those from the haematopoietic progenitor compartment (HPC). This allows stem cells to be selectively harvested free of other cell types, whereby the harvested cells may be cultured or otherwise manipulated.

BACKGROUND

Stem cells are primitive cells which are capable of self-renewal, and ultimately become differentiated into specific cell types of defined function. Stem cells are capable of proliferation either to generate further identical stem cells, or to produce more differentiated cell types. Later more differentiated cell types always become more differentiated on proliferation. Stem cells exist for most tissue types but are continuously active in the skin and mucosal systems, and for the blood and bone marrow (haematopoietic stem cells).

Haematopoietic stem cells are capable of self-renewal, multilineage proliferation and differentiation, and long-term support of the haematopoietic and lymphoid systems. They form a subpopulation within the Haematopoietic progenitor compartment (HPC), which mainly comprises cells of more limited potentiality. HPC cells are mainly located within the bone marrow stroma, where complex interaction with stromal cells, extracellular matrix components and cytokines, permits regulation of cell proliferation and differentiation. HPC cells are also present in the blood under a variety of physiological, pathological and iatrogenic circumstances. HPC can be harvested from bone marrow or peripheral blood, and will re-engraft the bone marrow following intravenous infusion in patients who have received ablative (i.e. destructive) doses of chemotherapy and/or radiotherapy, leading to regeneration of haematopoiesis and immunity. Thus, HPC cell transplantation is of considerable clinical utility in the management of patients with haematological and solid malignancies, bone marrow failure, and inborn errors of haematopoiesis, immunity or metabolism.

There is thus a need for supplies of autologous HPC cells which may be cultured in vitro prior to reintroduction into a patient whose HPC cell population has been depleted due to chemotherapy and/or radiotherapy. The populations of such HPC cells may take many weeks or months to recover naturally to their normal levels. The use of autologous cells from the patient himself avoids rejection of the transplanted cells. An object of the present invention is to address the culturing of such HPC cells in a convenient manner. This is carried out with a view to improving the rate and durability of haematopoietic recovery, the removal of any neoplastic contamination, and the possible use of HPC cells as a vehicle for immunotherapy and gene therapy.

In vivo, HPC cells are generally located within the bone marrow stroma. In vitro, HPC cells are able to adhere to bone marrow stromal layers before proliferating and releasing more committed progenitors. Stem cells undergo marked proliferation and differention into multiple lineages, ultimately giving rise to fully differentiated cells, such as red blood cells, platelets, a variety of white blood cells, and also immune cells such as T lymphocytes and B lymphocytes. Thus, the reintroduction of HPC cells or stem cells into the patient who is depleted therein, allows efficient repopulation of these haematopoietic cell types.

Although stromal layers may provide a suitable substrate for HPC cell immobilization and culture, such stromal layers

have a number of disadvantages. Firstly they are fragile. This imposes limitations on the types of systems which may be employed to culture such cells. The growth of HPC cells in vitro requires a rigid substrate on which the layers of stromal cells can be grown in order to maintain the integrity of the stroma. Moreover, it is found that HPC cells grown on stroma in this way only have a limited storage lifetime, of about six to eight weeks due to death of the stromal cells. A further problem is that the use of the stromal cells for the growth of HPC cells is limited to HPC cells derived from bone marrow, and cannot be used to culture other HPC cell types, such as mucosal cells.

In the case of an autologous graft, there may be a need to culture HPC cells for re-engrafting using stromal cells which are also derived from the patient himself. Immunological problems of rejection may arise if non-autologous stromal cells are used to culture the stem cells. The need to first collect and then grow a layer of the patient's stromal cells before they can be used to culture his HPC cells adds to the time and complexity of the production of the autologous HPC cells. In the case of bone marrow, a sample thereof contains not only stem cells but also the required stromal cells. However, if stem cells are harvested from peripheral blood or cord blood stromal cells are absent and have to be provided from a non-autologous source, leading to possible immunological problems.

Finally, the stromal cultures contain an ill-defined set of cells, growth factors etc. which renders the controlled culturing thereof very difficult if reproducible stromal cultures or predictable characteristics are to be obtained.

It is an object of the present invention to mitigate these problems.

SUMMARY OF THE INVENTION

Broadly speaking, the present invention resides in the discovery that a suitable substrate, such as a plastics material, can be coated with a fibrin matrix together with a substance capable of binding stem cells such as fibronectin, and that this will in turn selectively bind stem cells and allow culturing and manipulation thereof. Thus, the use of stromal cells may be avoided.

Specifically, one aspect of the present invention provides a system for selectively immobilizing stem cells, for example, those from the haematopoietic progenitor compartment (HPC), which comprises a substrate having a coating comprising a fibrin matrix, together with a substance capable of binding to the fibrin matrix and having a binding site for binding an RGD amino acid sequence for binding to the stem cells.

The binding substance capable of binding to the fibrin matrix and also having an ROD amino acid sequence may be a blood clotting factor such as fibronectin or thrombospondin or mixtures thereof, both of which bind to fibrin and also selectively bind stem cells via the RGD binding site on the binding substance. Alternatively the binding substance may be a synthetic molecule which includes an RGD sequence.

The specificity of binding of stem cells to a binding substance such as fibronectin allows the stem cells to be selectively immobilized on the substrate where they can be cultured or otherwise manipulated. This allows a variety of substrates including non-rigid flexible substrates which would not otherwise bind stem cells, to be employed.

In fact, in addition to stem cells early progenitor cells may also immobilise on the substrate so that these may also be selectively immobilized (and are included in the definition of the term "stem cells"). However, late progenitor cells will

not adhere to the coating on the substrate. Early progenitor cells retain some ability for multilineage proliferation, whereas late progenitor cells are generally restricted to a single lineage.

The nature of the substrate will vary dependent on the manipulations which are to be carried out on the immobilized stem cells. For example, selective binding of stem cells to the substrate allows for separation of stem cells from neoplastic cells. Thus, re-engrafted material may be freed from neoplastic cells prior to reintroduction into the patient so as to avoid a recurrence of the cancer.

Moreover, the immobilized stem cells may function as a target for gene transfection. There is evidence which suggests that incubation of HPC stem cells with fibronectin fragments improves the frequency of gene transfection using retroviral vectors. Such gene therapy generally involves the introduction of a gene either to introduce a new gene or to correct a genetic deficiency. Such therapy may be used to treat congenital defects of haemopoiesis, immunity and metabolism, or as a cancer therapy.

The invention thus avoids the use of layers of stromal cells, which require a rigid substrate to maintain their integrity.

Whilst it may be possible to culture stem cells in suspension, this tends to lead to early differentation of the cells; whereas the culturing of stem cells adhered to a substrate may allow early differentation to be avoided. Generally, we prefer to maintain a high population of stem cells for re-engrafting, which may then differentiate according to the patient's requirements in vivo.

A particularly preferred embodiment of the present invention, employs a closed container, such as a plastics bag formed of a flexible plastics material such as that of the type normally used in blood bags and which is gas permeable to carbon dioxide and oxygen so as to allow cell culture within the bag. However, such blood-bag plastics materials whilst suitable from the gas permeability point of view, are designed so as to be non-adherent to normal blood cell types so as to avoid the undesirable retention of blood cells on the inside of the bag. Moreover, such plastics materials conventionally include plasticiser materials and other reagents which may prove toxic or inhibitory to stem cell replication. Finally, the optical properties of such bags are preferably such as to allow the development of optical or spectoscopic cell and infection monitoring systems, thereby reducing the need for sub-sampling.

It is surprisingly found according to the present invention that such bags can be effectively coated with fibronectin or other binding substance, such that the fibronectin forms part of an adherent coating over the plastics surface, by employing a fibrin matrix which adheres to the plastics surface. Furthermore, it is surprisingly found that the stem cells are able to grow on the coating of fibronectin or other binding substance. This enables the advantageous culturing of autologous stein cells within sterile closed plastics containers, such as bags intended for blood storage. Procedures for handling and storing such bags are well established. Suitable bags are available from Tuta Laboratories (Australia) Pty Ltd., Sydney, Australia, under the trademark TUTA CLX. Suitable plastics materials include polystyrene and polyvinylchloride, which are often plasticised with TEHTM (tri(2-ethylhexyl)trimellitate) or DEHP (di(2-ethylhexyl)phthalate. Other suitable plastics materials are available from Baxter Health Care Ltd., Newbury, United Kingdom.

The use of a sealed plastics bag, such as those currently used for storing blood provides a convenient vehicle for culturing stem cells. Advantages include maintenance of sterility, reduced operator exposure to potential hazards from the cell harvest, and also from any reagents used during manipulative operations on the cultured cells.

Another important feature of the invention is not only the ability to selectively bind stem cells onto the coating of fibronectin or other binding substance, but also to allow their removal thereof at a chosen time. Suitable methods include the use of divalent cation chelation and competitive removal using RGD-containing peptides which will compete for the stem cell binding sites on fibronectin.

The limited ability of fibronectin or other binding substance to bind to the substrate is substantially enhanced by coating the substrate with a fibrin matrix. In a preferred embodiment, the fibrin matrix is produced in situ by the reaction of fibrinogen with thrombin whereby fibrin is deposited. The coating procedure may include the preliminary coating of the substrate with a fibrin matrix, followed by deposition thereon of fibronectin. Alternatively, the fibronectin may be incorporated into the fibrin matrix by depositing a mixture of fibrinogen and fibronectin on the substrate followed by treatment with thrombin to convert fibrinogen to fibrin in situ. In fact, fibrinogen is commonly available from lyophilised cryoprecipitate (obtained during blood protein fractionation) which also contains amounts of fibronectin. Fibrin-fibronectin coatings show good stem cell adhesion.

The fibrin matrix is also capable of being sterilised by conventional sterilisation techniques without substantial degradation.

A benefit of the system according to the present invention when used for culturing stem cells, is that it has general applicability to stem cells of all types, that is to say skin, mucosal, blood and marrow types (including HPC cells). In contrast, the use of layers of stromal cells to culture stems cells is applicable only to HPC cells derived from blood and bone marrow.

The coating may include other factors which nay assist the selection, culturing or release of the stem cells. For example, proteoglycans such as heparan sulphate, immobilized cytokines or modified monoclonal antibodies may be included. The binding selectivity of the coating may be enhanced by the inclusion of monoclonal antibodies directed to unique stem cell surface markers such as CD34, or antibodies which include an RGD binding site. The coating may include agents which will specifically bind and kill neoplastic cells. The skilled man will be aware of the need to provide other factors to enable the culturing of the immobilized stem cells.

DESCRIPTION OF PREFERRED EMBODIMENTS

Embodiments of the present invention will now be described by way of example only.

EXAMPLE 1

Cell Adhesion Molecule Expression by Human Haematopoietic Cell Line KGla

In order to validate the use of cell line KGla as a model system for HPC cells (including HPC stem cells), we characterised the pattern of cell adhesion molecule expression on HPC cells derived from normal bone marrow and peripheral blood following mobilisation with high-dosage cyclophosphamide and recombinant human granulocyte colony stimulating factor, and compared it with expression

from KGla. Samples were separated by Ficoll discontinuous density-gradient centrifugation. The mononuclear cell layer was removed, and the cells washed twice in a handling medium (phosphate buffered saline [PBS] containing 1% bovine serum albumin, 0.1% sodium azide and 0.02% ethylene diaminetetra-acetic acid [EDTA]). The cells were incubated for 15 min in 0.1% human gamma globulin (Sigma) in PBS to effect Fc receptor blockade. Aliquots of 5×10^5 cells per test were incubated with purified monoclonal antibody to one of a panel of cell adhesion molecules (Table I) for 15 min. The cells were washed twice in handling medium, and incubated with the second step reagent —a sheep anti-mouse R-phycoerythrin conjugate (Sigma), again for 15 mins. The cells were washed again, incubated for 15 min with mouse serum, to blockade the second-step reagent, and then incubated with the third step reagent—an anti-CD34 monoclonal antibody directly conjugated to fluorescein isothiocyanate (BG12-FITC, Becton Dickinson). The CD34 cell surface antigen identifies the HPC cells (which typically constitute only about 1% of the total cells). The cells were washed a final time, and fixed in 2% paraformaldehyde (Sigma) in PBS, prior to analysis. Appropriate control samples were established using monoclonal antibodies to CD45 as a positive control, and an isotype-specific antibody of irrelevant specificity as negative control, appropriately stained with each fluorochrome.

30–50,000 cells were acquired through a lymphoblastoid acquisition gate using a FACScan flow cytometer (Becton Dickinson), with prior standardisation of the instrument settings and two-colour compensation using control samples. Data was acquired and analysed using Lysis II software (Becton Dickinson). We demonstrated expression of ICAM-1, PECAM-1, LFA-3, LFA-1, VLA-4, VLA-5, L-Selectin and HCAM (Table II). Peripheral blood HPC demonstrated a similar pattern of expression, except in that there was less expression of LFA-3, and VLA-5. VLA-4 and VLA-5 are members of the B_1 integrin family, and recognise and adhere with high-avidity to fibronectin. L-Selectin recognises carbohydrate moieties such as Sialyl Lewis X, with low avidity binding. HCAM recognises collagen type 1 and hyaluronic acid. The lower levels of VLA-5 expression by circulating progenitors is suggestive that the VLA-5: fibronectin adhesion pathway may be instrumental in mediating HPC-stromal adhesion.

In view of the problems associated with obtaining highly purified human HPC populations, we therefore utilised the primitive human haematopoietic cell line KGla as a model early progenitor during development of a binding assay. Dual immunocytometry revealed KGla to be CD34 positive, and to express a similar cell adhesion molecule profile to normal haematopoietic progenitors (Table II).

EXAMPLE 2

Development of a Functional Binding Assay to Assess Adhesion of Haematopoietic Progenitors to Extracellular Matrix Components

We explored the functional binding of KGla to a panel of bone extracellular matrix components using a ^{51} chromium radiolabelling assay adapted from the work of Cheryl Hardy and Jose Minguell (1993). 1×10^7 KGla were incubated with 200 kBq ^{51}chromium (Amersham International) in 100 μl of foetal calf serum [FCS] for 1 hr. and washed twice in large volumes of Iscove's Modified Dulbecco's Medium [IMDM], with 10% FCS. The radiolabelled cells were aliquoted at a concentration of 2×10^5/200 μl, and dispensed into 2cm^2 wells of a 24 well flat-bottomed tissue culture dish (Costar). The wells were prior coated with extracellular matrix components known to be present in bone marrow stroma (Gordon, 1988; Clark, Gallagher & Dexter, 1992) (Table III). 100 μl of a solution containing 100 μg/ml (collagens) in 0.1% acetic acid in distilled water, or 50 μg/ml (fibronectin and proteoglycans) in distilled water, was added to each well and allowed to dry in a 37° C. oven for 1 hr. 1% denatured bovine serum albumin (Sigma) was used to coat negative control wells. The cells were incubated with the substrate for 2 hrs at 37° C., and the supernatant removed. The wells were washed twice with IMDM 10% FCS, and the washes and supernatant added to a scintillation vial. 0.5 ml of 0.1% Non-Idet in distilled water was added to each well for 15 min to lyse the adherent cells. This was also removed into a scintillation vial. Both adherent and non-adherent fractions were counted for 30 min in a Hewlett-Packard Gamma Counter, and the percentage binding calculated. Each assay was carried out in triplicate within each plate, and the mean percentage binding calculated. This assay system was standardised, and its reproducibity demonstrated. KGla were found to bind to tissue culture grade plastics (for comparison purposes), but not to TUTA CLX blood-bag plastic. The former was blocked by coating the well with denatured BSA or other proteins. KGla bound significantly to plasma and tissue fibronectin, but not to various collagens or proteoglycans (Table IV). Percentage adherence to fibronectin was remarkably constant over a range of cell concentrations between 1×10^4 to 2×10^6 cells per 2cm^2 well, but fell sharply at 1×10^7 cells/well, consistent with a maximal binding capacity of 430,000 cells/cm^2. Whether the maximal binding capacity is a reflection of saturation of fibronectin binding sites or of steric hinderance is currently unclear. Binding was abrogated at 4° C., but stable at room temperature and 37° C. The use of various incubating media (IMDM or RPMI, with and without FCS) made no difference to binding. In addition, binding reached a plateau after 1 hr, with no benefit to prolonged incubation periods. Fibronectin binding could be abrogated by carrying out the substrate incubation in a medium from which divalent cations had been removed (Hanks Balanced Salts Solution (Northumbria Biologicals) with 10 mmol EDTA (pH 7.01), or in a 3 mmol solution of an arginine-glycine-asparatate-serine peptide (Sigma) in IMDM (pH 7.01), though not in a solution of IMDM with 3 mmol of a control peptide (arginine-glycine-glutamate-serine) (Sigma). Finally, it was found that it made no difference to the % KGla binding whether the fibronectin was dried onto the well surface, or simply incubated at room temperature for 1 hr.

EXAMPLE 3

Optimal Coating TUTA CLX Blood Bag Plastic with Fibronectin

The possibility of coating a flexible plastics material was investigated. A TUTA CLX blood bag was cut open along the side seams, and a section of one side wag clamped in a Bio-Dot (Bio-Rad) dot blot apparatus so that the inner bag surface was exposed at the bottom of the 96 wells of the apparatus (96 well plate layout). Human plasma fibronectin [pFN] (Sigma) and tissue fibronectin [tFN] derived from foreskin fibroblasts (Sigma) were dissolved in pyrogen-free water to give a stock solution at 500 μg/ml. 50 μl of doubling dilutions of the pFN and tFN solutions were added across well columns 1–11. Column 12 contained water only (control). After 1 hr at room temperature, the well contents were removed, and the wells were washed 4 times with pyrogen-free water. Rabbit polyclonal anti-plasma fibronec-

tin [RPpFN] (Sigma) and mouse monoclonal anti-tissue fibronectin [MMtFN] (Sigma) were diluted 1:500 in buffer 1 (phosphate buffered saline (pH 7.2) containing 0.1% Tween 20, and 0.05% sodium azide), and 50 μl was added to each well of the microplate. After 90 min. at room temperature the well contents were removed, and wells were washed with buffer 2 (phosphate buffered saline (pH 7.2) containing 0.1% Tween 20, 0.05% sodium azide, 1% bovine serum albumin, and 4% polyethylene glycol). Alkaline phosphatasc conjugated goat anti-rabbit 1gG [APaR] (zymed) and alkaline phosphatase conjugated rabbit anti-mouse Ig [APaM] were diluted 1:1200 in buffer 2. 50 μl was added to the appropriate well series, and after 120 min at room temperature the well contents were removed, and the wells washed 5 times with buffer 2 and a further thrice with pyrogen-free water. The top manifold of the Bio-Dot apparatus was unclamped, and washed with sodium hydroxide, detergent (Decon) and distilled water to remove/destroy any of the above reagents which may have attached to the manifold. The manifold was clamped back into position over the sheet of blood bag plastic, and alkaline phosphatase substrate solution (Sigma) added at 140 μl per well. Colour could be seen to be developing at the well bottom, at the blood bag surface. The apparatus was left on an orbital mixer for 40 min at room temperature, 100 μl was transferred from each well to a half area microplate (Costar), and the optical density was read at 405 nm (minus 650 nm reference). The results are illustrated graphically in FIG. **1**. There was some background binding or the alkaline phosphatase labelled anti Ig antibodies (APaR and APaM) to fibronectin alone (3,6). The anti-tissue fibronectin antibody (MMtFN) showed relatively weak selective binding to pFN (2), and only slightly better binding to tFN (4). This may be a reflection of the particular concentrations of the anti-tFN antibody (MMtFN) and the anti-mouse Ig antibody (APaM) used in this experiment. The anti-pFN antibody (RPpFN) showed strong selective binding to both pFN (1) and tFN (5), and showed that binding of tFN to the plastic surface was superior to that of pFN at lower concentration ranges (0.5 to 8 μg/ml). Both pFN and tFN achieved saturation binding at approximately 20 μg/ml, but showed some evidence of "prozone" above approximately 50 μg/ml, i.e. there was some decrease in binding suggesting less firm anchorage of fibronectin to plastic at higher coating concentrations. This usually suggests multivalency of binding, such that more valencies are used at lower concentrations to establish firm binding while at higher concentrations there is competition for binding sites, less valencies are occupied, and the reagent is more readily detached.

EXAMPLE 4

Binding of KGla to Fibronectin Coated TUTA CLX Blood-Bag Plastic

It proved necessary to adapt the ^{51}chromium adhesion assay in order to examine KGla (used as a model for HPC cell binding) adhesion to TUTA CLX blood-bag plastic. The base of a 24 well plate was removed, a blood-bag cut open, and the internal surface adhered to the base of the plate. Care was taken to ensure that adhesive did not spread onto the test surface, and that a water-seal was achieved around each well individually. Wells were tested for continence with 1 ml of distilled water.

In an initial series of experiments, KGla adhesion was examined to the plastic itself, following coating with plasma or tissue fibronectin, or following coating with BSA. The results are summarised in Table V. KGla showed no significant binding to any of wells under these conditions, and we suspect that the negligable charge on the surface of the blood-bag plastic leads to only a very loose coating with fibronectin.

We considered a number of options to increase the extent and avidity of fironectin binding, including pre-coating the surface with a fibrin glue. Fibrin glue consists of two proteinaceous compounds, which form an adhesive matrix on reconstitution and mixing. one component is prepared from cryoprecipitate obtained from a large pool of voluntary UK blood donations. The material is lyophilised and subjected to heat treatment (80° C. for 72 hrs) to minimise the risk of viral transmission. The lyophilised croprecipitate consists mainly of fibrinogen (225 mg/vial) and factor XIII (50 units/vial), but probably also small amounts of other plasma proteins such as fibronectin, thrombospondin and von Willebrand's factor. The solvent for this component is water containing 20 mM Tris buffer (pH 7.5), and aprotinin (Trasylol, Bayer) at 3,000 kallikrein inactivator units/ml. The latter acts as a proteolytic enzyme inhibitor. The second component is lyophilised human thrombin, at 1000 IU/vial, reconstituted with a solution of 4 mM calcium chloride. When the two components are mixed, the thrombin activates the fibrinogen to fibrin, and also activates factor XIII to factor XIIIa, which stabilises the fibrin though cross-linking. Other proteins which may be present (such as fibronectin) will also be cross-linked into the matrix by factor XIIIa.

We coated TUTA CLX blood-bag plastic with fibrin matrix alone, fibrin matrix onto the surface of which plasma fibronectin had been incubated using the standard protocol (fibronectin contains fibrinogen binding domains), and fibrin matrix into which fibronectin had been added (to the fibrinogen component at 0.05 mg/ml) such that the fibronectin was cross-linked into the matrix by factor XIIIa. The results are tabulated in Table V.

TABLE I

Cell adhesion molecules studied.

Cytoadhesion molecule (CD)	Ligand	Monoclonal Antibody (Source)
Immunoglobulin Gene Superfamily		
ICAM-1 (CD54)	LFA-1	84HTO (Immunotech)
PECAM-1 (CD31)		5.6E (Immunotech)
LFA-3 (CD58)	LFA-2	AICD58 (Immunotech)
Integrin Family.		
β_1 VLA subfamily		
VLA-4 (CDw49d/CD29)	Fibronectin	HP2/1 (Immunotech)
VLA-5 (CDw49e/CD29)	Fibronectin	SAM1 (Immunotech)
β_2 leukocyte adhesion subfamily		
LFA-1 (CD11a/CD18)	ICAM1/2	IOT16 (Immunotech)
β_3 cytosdhesion subfamily		
Vitronectin receptor (CD51/CD61) Selectin Family.	Vitronectin	AMF7 (Immunotech)
L-Selectin Proteoglycan Analogues.	Carbohydrate	Dreg56 (Immunotech)
HCAM (CD44)	Collagen Hyaluronic Acid	F10-44-2 (Serotech)
CD36/LIMP II Family.		
Thrombospondin receptor (CD36)	Collagen Thrombospondin	FA-152 (Immunotech)

TABLE II

Cell adhesion molecule expression.

Adhesion molecule	Bone Marrow	Peripheral Blood	KG1a.
ICAM-1 (CD54):	91 ± 11%	93 ± 11%	98.6 ± 3%
PECAM-1 (CD31):	91 ± 7%	94 ± 6%	30 ± 13%
LFA-3 (CD58):	65 ± 25%	27 ± 18%	98 ± 1%
LFA-1 (CD11a):	84 ± 12%	78 ± 15%	99 ± 1%
VLA-4 (CDw49d):	67 ± 25%	71 ± 23%	99 ± 3%
VLA5-(CDw49e):	62 ± 19%	32 ± 19%	99 ± 3%
VNR (CD51):	9 ± 7%	5 ± 6%	7 ± 3%
L-Selectin:	64 ± 22%	60 ± 28%	5 ± 4%
HCAM (CD44):	98 ± 2%	97 ± 5%	99 ± 0.5%
CD36:	20 ± 11%	14 ± 14%	35 ± 13%

TABLE III

Extracellular matrix components studied.

Collagen Type I, acid soluble from human placenta. (Sigma, Type VIII)
Collagen Type III, acid soluble from human placenta. (Sigma, Type X).
Collagen Type IV, acid soluble from human placenta. (Sigma, Type VI)
Fibronectin, from human plasma. (Sigma).
Fibronectin, from human foreskin fibroblasts. (Sigma).
Heparan Sulphate, sodium salt from bovine kidney. (Sigma).
Chondroitin Sulphate A. sodium salt from bovine trachea. (Sigma).
Hyaluronic Acid, from human umbilical cord. (Sigma).
Glordon, 1988; Clark, Gallagher & Dexter, 1992)
Thrombospondin.

TABLE IV

KG1a binding to extracellular matrix components.

denatured albumin	8.3 ± 5.1%
collagen type I	5.2 ± 1.7%
collagen type III	10.1 ± 7.9%
collagen type IV	4.3 ± 2.1%
heparan sulphate	4.5 ± 1.2%
chondroitin sulphate	5.2 ± 1.7%
hyaluronic acid	6.9 ± 1.3%
plasma fibronectin	28.3 ± 5.2%
thrombospondin	76.2 ± 12.8%

TABLE V

KG1a binding to TUTA CLX blood-bag plastic.

substrate	mean % adherence ± 1 standard error	(number of experiments)
plastic	7.5 ± 2.3%	(3)
plasma fibronectin	7.4 ± 0.5%	(3)
tissue fibronectin	7.5 ± 1.9%	(2)
bovine serum albumin	5.0 ± 1.2%	(2)
fibrin matrix	22.9%	(1)
fibrin matrix + pFn (coated)	38.9%	(1)
fibrin matrix + pFn (inclusive)	32.5 ± 16.1%	(3)

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We claim:

1. A system for selectively immobilizing and culturing stem cells onto the inner surface of a flexible container, the system comprising a closed container formed of a flexible plastic material which is permeable to carbon dioxide and oxygen, the container including a substrate having a coating disposing a fibrin matrix, together with a substance capable of binding to the fibrin matrix and having an RGD amino acid sequence for binding to the stem cells, wherein the container is configured to allow stem cell culture to adhere to the inner surface of the flexible container.

2. A system according to claim 1 wherein the substance capable of binding to the fibrin matrix and also having an RGD amino acid sequence is fibronectin.

3. A system according to claim 1 wherein the substance capable of binding to the fibrin matrix and also having an RGD amino acid sequence is thrombospondin.

4. A system according to claim 1 wherein the fibrin matrix is produced in situ on the substrate by the reaction of fibrinogen with thrombin.

5. A system according to claim 4 wherein the fibrinogen is in the form of cryoprecipitate, which also contains fibronectin.

6. A system according to claim 1 which further comprises a factor selected from the group consisting of proteoglycans, cytokines and monoclonal antibodies.

7. A system according to claim 1 which further comprises a layer of stem cells bound to the coated substrate.

8. The system of claim 1, wherein the closed container is a blood bag.

9. A method of harvesting stem cells from peripheral or cord blood, the method comprising the steps of introducing the blood into a closed container formed of a flexible plastic material which is permeable to carbon dioxide and oxygen, disposing in said container a substrate having a coating including a fibrin matrix, together with a substance capable of binding to the fibrin matrix and having an RGD amino acid sequence for binding to the stem cells, such that the stem cells are selectively immobilized on the substrate and harvested from the blood, and adhering said stem cells to the inner surface of the flexible container.

10. A method of storing and preserving stem cells on the inner surface of a flexible container, which comprises the steps of storing the stem cells in a closed container formed of a flexible plastic material which is permeable to carbon dioxide and oxygen, the closed container including a substrate having a coating including a fibrin matrix, together with a substance capable of binding to the fibrin matrix and having an RGD amino acid sequence for binding to the stem cells such that the stem cells are immobilized on the substrate within the container, and adhering said stem cells to the inner surface of the flexible container.

11. The method of claim 10, wherein the stem cells are from peripheral or cord blood.

12. A method of transfecting stem cells which comprises maintaining the stem cells in a closed container formed of a flexible carbon dioxide-permeable and oxygen-permeable plastic material, the container including a substrate having a coating including a fibrin matrix, together with a substance capable of binding to the fibrin matrix and having an RGD amino acid sequence for binding to the stem cells, such that the stem cells are immobilized on the substrate within the container, and adhering said stem cells to the inner surface of the flexible container;

and transfecting the stem cells with a vector containing a heterologous gene.

13. A method according to claim 12 wherein the vector is a retroviral vector.

14. A method according to any of claims 9, 10, 12, or 13 wherein said substance is fibronectin or thrombospondin.

15. The method of claim 12, wherein the stem cells are from peripheral or cord blood.

16. A method of culturing stem cells in vitro onto the inner surface of a flexible container, which comprises the steps of culturing the stem cells in a closed container formed of a flexible carbon dioxide-permeable and oxygen-permeable plastics material, the container including a substrate having a coating disposing a fibrin matrix, together with a substance capable of binding to the fibrin matrix and having an RGD amino acid sequence for binding to the stem cells, such that the stem cells are immobilized and cultured on the substrate within the container, and adhering said stem cells to the inner surface of the flexible container.

17. The method of claim 16, wherein the coating further comprises a factor selected from the group consisting of proteoglycans, cytokines and monoclonal antibodies.

18. The method of claim 16, wherein the stem cells are from peripheral or cord blood.

* * * * *

EXHIBIT F

US005686304A

United States Patent [19]

Codner

[11] Patent Number: 5,686,304

[45] Date of Patent: Nov. 11, 1997

[54] **CELL CULTURE APPARATUS AND METHOD**

[75] Inventor: **Meryl Codner**, St. Paul, Minn.

[73] Assignee: **Avecor Cardiovascular, Inc.**, Plymouth, Minn.

[21] Appl. No.: **574,452**

[22] Filed: **Dec. 15, 1995**

[51] **Int. Cl.**[6] **C12N 5/00**; C12N 5/02

[52] **U.S. Cl.** **435/325**; 435/383; 435/283.1

[58] **Field of Search** 435/240.1, 283.1, 435/240.25, 325, 383; 210/500.21; 428/411.1, 429, 447

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Assistant Examiner—Christopher R. Tate
Attorney, Agent, or Firm—Fredrikson & Byron, P.A.

[57] **ABSTRACT**

A cell culture apparatus formed of a plurality of thin (e.g., 0.005" or less), spaced, gas-permeable, silicone membranes sealed at their edges to form a bag-like vessel comprising one or more interior chambers suitable for containing cell culture media. A suitable portion of the membrane surfaces are of suitable thickness and surface area to provide structural integrity to the apparatus and sufficient gas permeability for cell growth within the chamber.

24 Claims, 5 Drawing Sheets

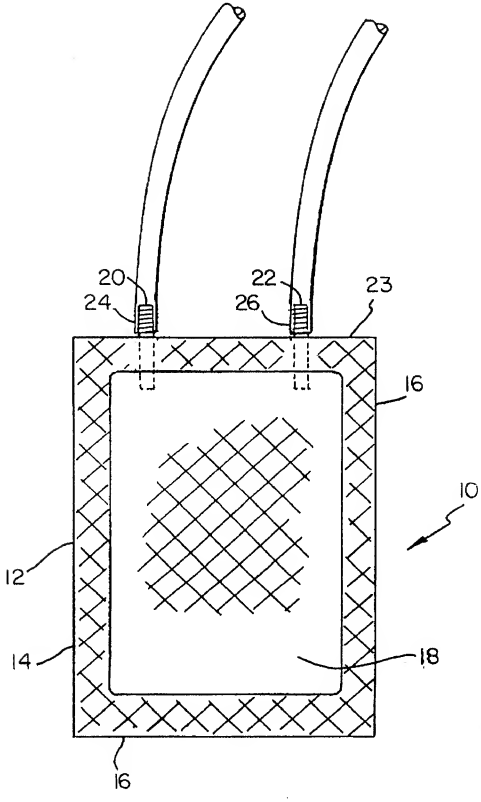

Fig. 1

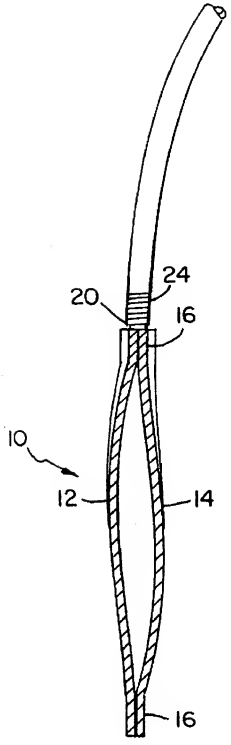

Fig. 2

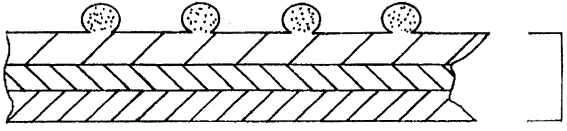

Fig. 3

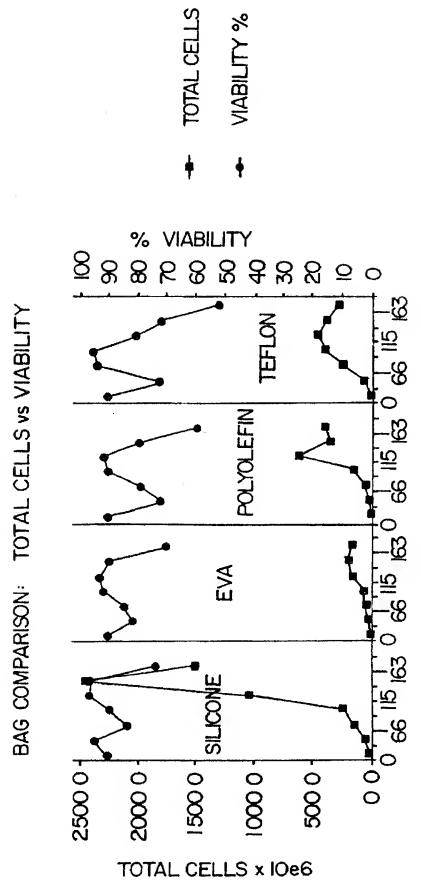
BAG COMPARISON: TOTAL CELLS vs VIABILITY
TOTAL CELLS
VIABILITY %
% VIABILITY
100
90
80
70
60
50
40
30
20
10
0
2500 0
2000 0
1500 0
1000 0
500 0
0 0
TOTAL CELLS x 10e6
SILICONE
EVA
POLYOLEFIN
TEFLON
0
66
115
163
TIME (HOURS)

Fig. 4

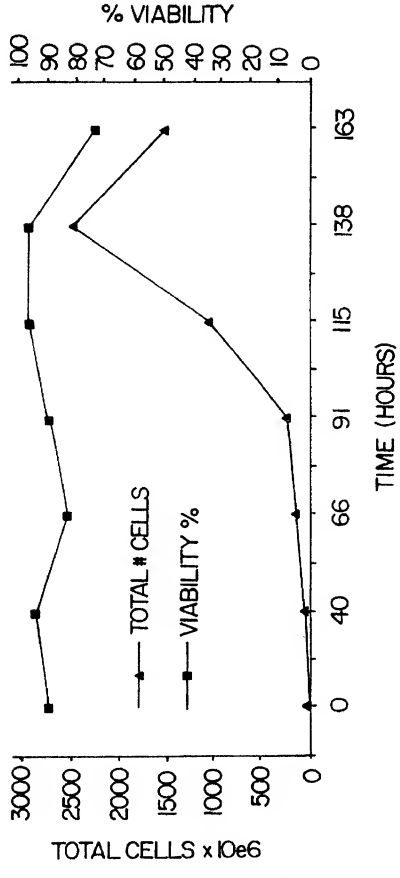
% VIABILITY
100
90
80
70
60
50
40
30
20
10
0
163
138
115
91
66
40
0
TIME (HOURS)
TOTAL # CELLS
VIABILITY %
3000
2500
2000
1500
1000
500
0
TOTAL CELLS x 10e6

Fig. 5

CELL CULTURE APPARATUS AND METHOD

TECHNICAL FIELD

The present invention relates to cell culture apparatuses and methods, and particularly to cell culture apparatuses in the form of cell culture bags and similar devices. In another aspect the invention relates to silicone membranes, such as those used to prepare oxygenators for biological fluids.

BACKGROUND OF THE INVENTION

The culture of mammalian cells and tissues is now quite common in the areas of molecular biology and biotechnology. Several textbooks provide guidance in the materials and methods involved in preparation, sterilization, and cell propagation. See, for instance, R. I. Freshney, Chapter 1, "Introduction to basic principles", in *Animal Cell Culture: A Practical Approach*, 2d Ed., IRL Press (1994).

A variety of cell types can now be grown in culture, including connective tissue cells, skeletal, cardiac, and epithelial cells, neural cells, endocrine cells, melanocytes, and many types of tumor cells. Similarly a variety of media are available, depending on the particular growth requirements of the cells and the growth conditions.

Depending on the type of cells, their intended use, and the conditions of growth, cells can be grown in a number of different configurations. Most cultures are propagated in the form of a monolayer, with the cells anchored to a glass or plastic substrate. Some, however, are preferably grown in suspension, which has the advantage of simpler propagation. Using suspension, subculture can be accomplished by simple dilution rather than by detaching (e.g., by trypsinization) the cells from anchored growth. Growth in suspension also provides increased surface area with increased bulk, as well as improved ease of harvesting, and the possibility of achieving a "steady state" culture.

Growth in suspension is hampered, however, by a number of factors that do not affect monolayer cultures. See, for instance, B. Griffiths, "Scaling-up of animal cell cultures", Chapter 3 in *Animal Cell Culture: A Practical Approach*, 2d Ed., IRL Press (1994), the disclosure of which is incorporated by reference.

Cell growth kinetics in suspension culture can be affected by a number of considerations. The effect of varying growth conditions can be important, such as the growth temperature, initial growth phase of cells, inoculation density, stirring rate, and medium surface area. So, too, can the selection of medium, nutrients, and pH all affect growth in a predictable, controllable manner.

The scale-up of animal cell cultures is particularly dependent on the ability to supply sufficient oxygen without causing cellular damage. Since oxygen is typically only sparingly soluble in culture media, it is often necessary to supply oxygen to the medium throughout the life of the culture. Typically, a culture can be aerated by one, or a combination, of the following methods: surface aeration, sparging, membrane diffusion, medium diffusion, increasing the partial pressure of oxygen, and/or increasing the atmospheric pressure. M. Schneider, et al., for instance, recently described the application of bubble-free aeration to animal cell culture processes to avoid shear stress and foaming often associated with direct sparging. (See "Bubble-Free Oxygenation by Means of Hydrophobic Porous Membranes", *Enz. Microb. Tech.*, 17(9):839 (1995)).

Typically, cell culture production of either cells or cell-secreted products begins with the small scale growth of

cells. Traditional vessels for small volume cultures include multi-well plates, T-flasks, roller bottles and spinner flasks. Most small-scale cultures are limited to 5 to 7 days of growth. Final cell densities and total cell numbers are frequently low due to space, surface area and gas exchange limitations. Thus, it is frequently necessary to use multiple vessels to grow a desired cell population. The use of multiple vessels, however, means increased labor and contamination risk due to the need to open, close, fill and harvest from each vessel.

In recent years a number of manufacturers have also begun to offer cell culture devices in the form of flexible, disposable bags formed of biologically inert and gas-permeable plastic materials such as fluoroethylenepropylene copolymers. U.S. Pat. Nos. 4,847,462 and 4,945,203 (Soodak), for instance, both relate to methods and apparatuses for manufacturing air and water tight bags of plastic films, the bags being useful for culturing living cells. Cell culture bags said to be covered by these patents are available from American Fluoroseal Corporation (Columbia, Md.).

Still other patents describe the use of flexible bags as components of cell culture media systems. See, for instance, U.S. Pat. Nos. 4,937,194 and 4,829,002 (Pattillo, et al.); 5,350,080 (Brown); and 5,362,642 (Kern). Yet other patents relate to cell culture devices that involve the use of oxygen-permeable materials. GB Pat. No. 2268187 (Armstrong, et al.), for instance, describes a vessel having part of its walls formed as a gas-permeable membrane. The vessel may be a petri-dish whose base consists of the membrane, such as silicone rubber. Alternatively, it may be a Roux bottle in which a side wall comprises the gas-permeable membrane.

GB Pat. No. 2255571 (Burr, et al.) describes a method of cell culture involving placing cells in a culture medium on a silicone membrane and periodically feeding the cells, and JP 2234670 describes the use of a shell membrane as the earlier for culturing an adhesive animal cell.

Similarly, U.S. Pat. No. 4,661,455 (Hubbard) relates to a double membrane bag assembly for supplying nutrients and oxygen to growing cells. The device relies on the use of gas permeable membranes such as those formed of dimethyl silicone. U.S. Pat. No. 5,008,197 (Wergeland) describes an apparatus for cultivating cells that involves the use of an oxygen-permeable membrane (such as a "silicone membrane") to supply oxygen to the media. Finally, U.S. Pat. No. 5,288,631 (Baumgartner, et al.) describes a tubular membrane for the delivery of gaseous fluid to a surrounding medium, for use in oxygenating an animal cell reactor.

On a separate subject, gas-permeable silicone rubber membranes have themselves been described as useful for a number of products, such as blood oxygenators. The preparation and use of such silicone rubber membranes is described, for instance, in U.S. Pat. Nos. 3,489,647 (Kolobow I), 3,969,240 (Kolobow II) and 4,093,515 (Kolobow III and 3,510,387 (Robb). Such membranes can be provided either with an integral support material (e.g., with a fabric scrim) or in unsupported form. They can also be provided in a form where one or more of the silicone layers is compounded with a filler, such as fumed silica or carbon black.

As far as Applicant is aware, silicone rubber membranes have not previously been suggested or used for the purpose of fabricating cell culture devices in the form of bags or envelopes. Yet, what is clearly needed are new and improved means for culturing cells, particularly those that facilitate improved oxygen supply.

SUMMARY OF THE INVENTION

The present invention provides a cell culture apparatus comprising a cell culture bag having thin, gas-permeable

silicone rubber membrane walls. Preferably, the membrane is formed as a reinforced laminate of silica-filled rubber silicone layers, the laminate having a thickness on the order of 0.005 inches (0.127 mm) or less. An apparatus of the invention can provide gas exchange rates that are one or more orders of magnitude higher than most conventional cell culture bags. The higher gas exchange rate results in significantly higher cell densities and well viabilities.

In a preferred embodiment, the apparatus comprises a plurality of thin, gas-permeable, silicone rubber membranes sealed at their edges to form a waterproof bag-like vessel comprising one or more interior chambers suitable for containing cell culture media. The membranes are of suitable thickness and surface area to provide structural integrity to the apparatus and sufficient gas permeability to accommodate cell growth within the chamber.

The apparatus provides a surprising and optimal combination of such properties as gas (including O_2 and CO_2) permeability, structural integrity, optical transparency and clarity, temperature resistance, vapor transmission, resilience, temperature tolerance, low extractables, adaptability, and cost. The apparatus provides optimal spatial efficiency for use in propagating suspension cultures and microcarrier-attached cultures. The apparatus can be GMP manufactured, and is capable of being sterilized at the time of manufacture or at any time prior to use.

In a particularly preferred embodiment, the thickness of one or more membranes making up the vessel is on the order of 0.003" (0.076 mm) or less, and the apparatus further comprises a plurality of inlet/outlet ports flowably connected to one or more interior chambers of the vessel.

In another aspect, the present invention provides a method of preparing a cell culture apparatus as presently described. In yet another aspect, the invention provides a combination comprising a vessel of the present invention in combination with cell growth medium, as well as a combination of a vessel, medium, and cells. In a related aspect, the invention provides a method of culturing cells that involves the use of a cell culture apparatus as described herein.

BRIEF DESCRIPTION OF THE DRAWING

In the Drawing:

FIG. **1** shows a perspective view of a cell culture apparatus in accordance with the invention.

FIG. **2** shows a cross-sectional view of the apparatus shown in FIG. **1**.

FIG. **3** shows a magnified cross-sectional view of a membrane used to form the apparatus of FIG. **1**.

FIG. **4** shows a chart comparing total cell growth to cell viability, for culture bags fabricated from various materials.

FIG. **5** shows a chart comparing total cell growth to time, for culture bags of the present invention.

DETAILED DESCRIPTION

A preferred apparatus of the invention will be described with reference to the Drawing. In FIGS. **1** and **2** there is shown an apparatus **10**, formed of two opposing silicone membranes, designated **12** and **14**, respectively. The membranes are sealed together at their edges **16** to form a water-tight seal, and in turn, creating interior chamber **18**.

Shown also are inlet and outlet ports **20** and **22**, respectively, which provide for the flow of fluids (and/or gasses) through the top portion **23** of the apparatus and into chamber **18**. The inlet/outlet ports can be used for any suitable purpose, such as the delivery of media, cells, and/or gasses. They can also be used for removal of media, cells, waste or reaction products, and the venting or withdrawal of vapors or gasses. As shown in FIG. **1**, ports **20** and **22** are each preferably provided with spring-like barbs, in the form of internal coiled wires **24** and **26**, respectively, in order to provide additional support and flexibility in the course of repeated and prolonged use.

An apparatus of the invention can be prepared using materials and techniques within the skill of those in the respective art, given the present teaching. Preferably, the materials used to fabricate components of an apparatus are themselves inert and biocompatible (e.g., USP XXII Class VI biocompatible).

Silicone membranes for use in this invention can be obtained or prepared using conventional techniques for the preparation of gas-permeable membranes. Cross-linked silicone polymers of appropriate molecular weight provide elastomeric properties, and can be used to prepare either RTV rubbers or heat-cured rubbers. Suitable membranes can be obtained from a number of sources, and can be prepared by conventional techniques. See, for instance, "Silicones", pgs. 1048–1059 in *Concise Encyclopedia of Polymer Science and Engineering*, Kroschwitz, J. ed., Wiley & Sons, 1990.

Preferred membranes comprise an organosilicone rubber of the type described in U.S. Pat. Nos. 3,489,647 (Kolobow I), 3,969,240 (Kolobow II) and 4,093,515 (Kolobow III) and 3,510,387 (Robb), as well as in Robb, "Thin Silicone Membranes—Their Permeation Properties and Some Applications", *Ann. NY Acad. Sci.*, 146:119–137 (1968), the disclosures of each of which are incorporated herein by reference. Particularly preferred membranes are provided as a reinforced laminate of a plurality of silicone rubber layers, one or more of the layers being compounded with a filler (e.g., fumed silica).

Suitable reinforcing means are described, for instance, at col. 5, line 62 to 6, line 13 of the '515 patent. Suitable filler materials are selected from the group consisting of filler-free silicone rubber, silicone rubber compounded with silica filler, and silicone rubber compounded with carbon black filler are preferred. For routine cell culture applications, most preferred are those prepared using silica filler. Preferred rubber materials employ a filler in mounts up to about 30%, preferably up to about 40%, and more preferably up to about 50% by weight, based on the dried weight of the membrane.

Conventional silicone membranes are prepared using a conventional process involving the formation of a toluene dispersion and peroxide cure. Alternatively, membranes can be prepared using a more recently developed processes such as those involving a solventless liquid silicone starting material and a platinum cure. Platinum-cured membranes are preferred in a number of respects, since they will typically tend to be free of residual toluene and decomposition products that may be present in peroxide cured products. Peroxide-cured products are preferred, however, for their ability to resist the formation of pinholes or other imperfections in the course of extended handling and use.

Silicone membranes used to fabricate vessels of the present invention are preferably thin, in order to improve their gas transfer and other desirable properties. Surprisingly, membranes having a thickness of on the order of 0.005" (0.127 mm) or less provide an optimal combination of such properties as gas transfer and structural integrity for use in the present invention. Particularly preferred are those having a thickness of on the order of 0.003" (0.076 mm) or less.

The thickness of a membrane can be determined using conventional methods, e.g., by gravimetric or visual means. Preferably, the thickness of a membrane is determined by microscopic evaluation in the following manner. A membrane is placed on a clean microscope slide and cut with a sharp blade to the size and shape of the slide using the slide as a template. A second clean glass slide is applied to the opposite surface of the membrane, and the resulting sandwich is retained in the jaws of a small clamp or vice as a dye material is applied to the exposed edge of the membrane. The thickness of the membrane is visually determined using standard techniques, by comparison to the gradations within a calibrated "Filar" eyepiece when used with a suitable optical microscope (e.g., a Leeds microscope). With a reinforced membrane, the thickness is, determined as the thickness of the silicone laminate itself, as opposed to the periodic extensions formed by the reinforcing fabric. (See, e.g., the cross-sectional view of FIG. 3).

In the course of fabricating an apparatus of the present invention, silicone rubber membrane materials can typically be provided in bulk form, e.g., in rolls. Pieces of suitable size are cut and paired together to form a bag of desired dimensions. Ports and/or tubes can be placed in desired positions between the paired membranes, and the bags glued or welded shut by the formation of seams at their edges. In order to achieve optimal support, reinforced membranes are preferably positioned such that their nonreinforced surfaces face each other, and in turn, with their reinforced surfaces in an outward orientation.

Bags are preferably sealed by a method that provides a durable, waterproof seal yet retains the structural integrity of the bag. Examples of suitable sealing means include the use of a suitable adhesive (e.g., an room temperature vulcanizing "RTV") silicone glue), interspersed and cured between the opposing membrane surfaces. Examples of suitable adhesives are described, for instance, in Skeist (ed.), *Handbook of Adhesives*, 3rd Ed., Chapt. 30, "Silicone Adhesive Sealants and Adhesives", J. W. Dean, (1990). Alternative sealing means include the use of a clamping mechanism to hold two opposing membranes together. Another alternative provides that an integral membrane that is itself molded or formed into the shape of a bag-like vessel. Yet another alternative provides two opposing membrane surfaces that are configured to be sufficiently matable, as in the manner of a "zip-lock" like pressure fit seal.

Preferably, the membranes are sealed by the use of a medical grade, RTV silicone glue. For example, using two pieces of siliconc rubber membrane, a bead (approx. 1/16) of suitable glue was applied from a syringe to one surface (the non-reinforced surface) of one membrane, approximately 0.5 inches from the edge. A tube assembly was positioned along a short side of the membrane, and extra glue was applied around the tube assembly. The second, opposing membrane was applied over the glue and the glue was tapered until it became flattened (without working over the edge of the membrane). With a sheet of wax paper placed over the assembly, the apparatus was flattened (and worked around the tube assembly) with a roller, keeping the glue seam as straight as possible. The glue was allowed to fully cure, and the bag was used as a receptacle for media.

The component pieces, and/or bags themselves, can be cut to the desired dimensions either prior to or following the sealing process. Bags can be prepared, for instance, having any desired combination of dimensions, nominal maximum capacity, and total surface area. The nominal capacity of suitable culture bags will typically range between about 20 ml and about 1000 ml. Volumes less than about 20 ml tend to be more conveniently used in other culture formats, while volumes over about 1000 ml tend to exceed the strength limits of even reinforced silicone membranes.

Those skilled in the art will appreciate the selection of suitable port and attachable tubing materials available for such purposes. Preferably, the port and tubing materials are flexible, inert, pliable, durable, and capable of being sterilized using conventional techniques (e.g., autoclaving, ethylene oxide, gamma irradiation).

The inlet/outlet port can be provided in any convenient and suitable configuration, and tubing can be attached to the bag by a number of means. Typically, a single apparatus will provide one or more inlet ports for each compartment within the vessel. Each port can be fitted with quick connect couplers and attachment sites, either at the surface of the membrane or separated from it by a distance of flexible tubing extending outwardly from the edge. For instance, a short distance of tubing can be fitted on its distal end with a female luer lock and cap in order to facilitate its attachment to other tubing or devices. Ports and tubing are preferably of standard types and dimensions, e.g., to facilitate tubing sets having 3/8 inch inner diameter.

The bulk silicone membrane materials, and the apparatus fabricated from such materials, should be handled carefully in order to avoid the formation of pinholes, bubbles, cracks, delaminations and other structural or visible flaws or imperfections. An apparatus of the invention can be provided individually packaged in non-sterile but autoclavable pouches which can be sterilized by the user, for instance, at 121° C., at 1 atm for 20 minutes prior to use. Unsterilized pouches, in turn, are stable upon storage at ambient conditions.

An apparatus of the invention can be used to culture cells using materials, methods and techniques within the skill of those in the art. Typically, the apparatus will be sterilized and aseptically attached via its inlet port to a sterilized tubing assembly that, in turn, is aseptically attached to a sterile media filter. The filter, in turn, is attached to a sterile or non-sterile media reservoir. Closing the clamp on the outlet port of the apparatus, the reservoir is used to fill the apparatus to the desired level. When filled, the inlet port is closed or clamped shut, and both ports can be wiped with alcohol and covered.

The invention will be further described with reference to the following non-limiting Examples.

EXAMPLES

EXAMPLE 1

Cell Growth Comparisons

The following three commercially available polymeric bags were each compared with an apparatus of the present invention for use in cell growth using a standard hybridoma cell culture: Teflon bags (American Fluoroseal), polyolefin bags (Fenwal), and ethylene vinyl acetate copolymer ("EVA") bags (Stedim Co.). Due to differences in size or recommended volume, media volume and initial cell inoculum were normalized according to the surface area of the various bags. Bags were initially fried with 0.4 ml/cm^2 of medium (Dulbecco's Minimal Essential Medium High Glucose ("DMEM HG") supplemented with 10% fetal bovine serum ("FBS") and 6 mM glutamine) for each cm^2 of bag surface area. On day one, 0.15×10^6 cells/ml were added to each bag. All bags were placed in a 37° C. incubator at 85–90% humidity and place on a rocker set to agitate at 90

sec/cycle. The incubator was maintained at 10% CO_2 in air. Cell samples were taken daily. Each bag was fed with additional medium in order to maintain the cells in log phase until an upper media volume of 1.6 ml/cm^2 was reached. Total length of the study was 163 hours.

The results of the study are shown in FIG. 4. It can be seen that the silicone bag had at peak a cell density of 3.6×10^6 cells/ml with viability of 98%. This is over four-fold higher than the next closest bag. Total cells produced by the silicone bag at peak was 2.5×10^9 at 98% viability.

EXAMPLE 2

Maximum Cell Growth Study

A study was conducted using cell culture bags of the present invention. The objective of the study was to culture conventional hybridoma cells in order to achieve maximum cell count and viability with a minimum number of growth vessels. Hybridoma cells were grown in the manner described in Example 1, although using a 600 ml culture bag of the invention. The results of the study are shown in TABLE I below:

TABLE I

Time (hours)	Total # cells (× 10e5)	Viability (%)
0	25.95	92
40	64.01	96
66	162.62	85
91	262.96	91
115	1065.68	98
138	2491.20	98
163	1529.32	75

These results demonstrate that a 600 ml cell culture bag of the present invention provided approximately 2.5×10^9 cells with 98% viability at peak. Assuming the typical cell density achieved using a standard flask (e.g., of the "T-225" type) to be 0.7×10^6 cells/ml, it would require 36 flasks at 100 ml each to produce same number of cells. It is believed that the high gas exchange properties of the silicone bag can be attributed to the increased cell growth and viability. These results clearly show that the cell culture bag is excellent for production-scale inoculations.

EXAMPLE 3

Recombinant Protein Production

Recombinant protein production by mammalian cells is highly dependent on the overall cellular environment. It is known that as a cell population increases, the need for efficient control of dissolved oxygen and pH (via CO_2/$NaHCO_3$ buffering) is increased. Silicone membrane cell culture bags of the invention were evaluated to determine whether the higher gas exchange achievable using a bag of the present invention can provide the necessary level of O_2 and CO_2 using a standard, humidified CO_2 incubator.

A 1 liter Teflon bag, and a 1 liter bag of the present invention were each inoculated 1×10^5 cells/ml to final volumes of 400 ml per bag. Each bag type was cultured 10 days in a humidified CO_2 incubator with cell counts, viabilities, pH, glucose, lactic acid and recombinant protein measurements done daily. Results of pH and recombinant protein production in both μg/ml and μg per 10^5 cells are shown in FIG. 5.

Results clearly show elevated recombinant protein production with the silicone membrane bag in both μg/ml and μg per 10^5 cells. This increase in protein production is believed to be due to enhanced gas exchange across the silicone membrane. The silicone cell culture bag has gas permeability rates that are orders of magnitude higher than commercial bags formed of a Teflon-brand material. Evidence of increased CO_2 control in the silicone bag was seen in the pH control. The silicone bag was 0.57 pH units closer to control medium pH than the commercial bag at experiment end. The results clearly show that the silicone membrane bag was ideal for recombinant protein production by mammalian cells.

It is to be understood that the invention is not intended to be limited to the above embodiments, which are shown for purposes of illustration in the Drawing and described above, but is intended to include any modification or variation thereof falling within the scope of the appended claims.

What is claimed is:

1. An apparatus comprising a cell culture vessel comprising a thin, gas-permeable wall formed of a silicone rubber membrane, wherein the thickness of the membrane is about 0.003 inches (0.076 mm) to about 0.005 inches (0.127 mm) and the membrane is provided in the form of a reinforced laminate of filled rubber silicone layers the apparatus further comprising a plurality of inlet/outlet ports flowably connected to the vessel.

2. An apparatus according to claim **1** wherein the apparatus comprises a plurality of thin, gas-permeable, silicone rubber membranes sealed at their edges to form a waterproof vessel comprising one or more interior chambers suitable for containing cell culture media.

3. An apparatus according to claim **2** wherein the membranes are sealed together to form the vessel by the use of a medical grade silicone glue.

4. An apparatus according to claim **1** wherein one or more of the silicone rubber membranes have been compounded with silica filler at a concentration of up to about 50% by weight filler, based on the weight of the membrane.

5. An apparatus according to claim **4** wherein the silica filler is carbon black filler, at a concentration of up to about 30% by weight.

6. An apparatus according to claim **4** wherein the apparatus has a nominal capacity of between about 20 ml and about 1000 ml.

7. A method of preparing a cell culture apparatus comprising the steps of forming a thin, gas permeable silicone rubber membrane into the form of a wall of a cell culture vessel, wherein the thickness of the membrane is about 0.003 inches (0.076 mm) to about 0.005 inches (0.127 mm) and the membrane is provided in the form of a reinforced laminate of filled rubber silicone layers and providing the apparatus with a plurality of inlet/outlet ports flowably connected to the vessel.

8. A method according to claim **7** wherein the apparatus comprises a plurality of thin, gas-permeable, silicone rubber membranes sealed at their edges to form a waterproof vessel comprising one or more interior chambers suitable for containing cell culture media.

9. A method according to claim **8** wherein the membranes are sealed together to form the vessel by the use of a medical grade silicone glue.

10. A method according to claim **7** wherein one or more of the silicone rubber membranes have been compounded with silica filler at a concentration of up to about 50% by weight filler, based on the weight of the membrane.

11. A method according to claim **10** wherein the silica filler is carbon black filler, at a concentration of up to about 30% by weight.

12. A method according to claim 10 wherein the apparatus has a nominal capacity of between about 20 ml and about 1000 ml.

13. A combination comprising a cell culture apparatus comprising a cell culture vessel comprising a thin, gas-permeable wall formed of a silicone rubber membrane and a plurality of inlet/outlet ports flowably connected to the vessel, and (b) a medium for the growth of cells contained within the apparatus, wherein the combination provides a sterile environment for the introduction and growth of cells and wherein the thickness of the membrane is about 0.003 inches (0.076 mm) to about 0.005 inches (0.127 mm) and the membrane is provided in the form of a reinforced laminate of filled rubber silicone layers.

14. A combination according to claim 13 wherein the apparatus comprises a plurality of thin, gas-permeable, silicone rubber membranes sealed at their edges to form a waterproof vessel comprising one or more interior chambers suitable for containing cell culture media.

15. A combination according to claim 14 wherein the membranes are sealed together to form the vessel by the use of a medical grade silicone glue.

16. A combination according to claim 13 wherein one or more of the silicone rubber membranes have been compounded with silica filler at a concentration of up to about 50% by weight filler, based on the weight of the membrane.

17. A combination according to claim 16 wherein the silica filler is carbon black filler, at a concentration of up to about 30% by weight.

18. A combination according to claim 16 wherein the apparatus has a nominal capacity of between about 20 ml and about 1000 ml.

19. A method of culturing cells comprising the steps of

(a) providing a cell culture apparatus comprising a cell culture vessel comprising a thin, gas-permeable wall formed of a silicone rubber membrane and a plurality of inlet/outlet ports flowably connected to the vessel,

(b) adding a suitable combination of cells and medium to the chamber, and

(c) incubating the chamber under conditions suitable to propagate cell growth, wherein the thickness of the membrane is about 0.003 inches (0.076 mm) to about 0.005 inches (0.127 mm) and the membrane is provided in the form of a reinforced laminate of filled rubber silicone layers.

20. A method according to claim 19 wherein the apparatus comprises a plurality of thin, gas-permeable, silicone rubber membranes sealed at their edges to form a waterproof vessel comprising one or more interior chambers suitable for containing cell culture media.

21. A method according to claim 20 wherein the membranes are sealed together to form the vessel by the use of a medical grade silicone glue.

22. A method according to claim 19 wherein one or more of the silicone rubber membranes have been compounded with silica filler at a concentration of up to about 50% by weight filler, based on the weight of the membrane.

23. A method according to claim 22 wherein the silica filler is carbon black filler, at a concentration of up to about 30% by weight.

24. A method according to claim 22 wherein the apparatus has a nominal capacity of between about 20 ml and about 1000 ml.

* * * * *

EXHIBIT G

US005989215A

United States Patent [19]

Delmotte et al.

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[45] Date of Patent: Nov. 23, 1999

[54] **FIBRIN DELIVERY DEVICE AND METHOD FOR FORMING FIBRIN ON A SURFACE**

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[73] Assignee: **Baxter International Inc.**, Deerfield, Ill.

[21] Appl. No.: **08/679,658**

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Related U.S. Application Data

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[30] **Foreign Application Priority Data**

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[51] **Int. Cl.**[6] **A61M 37/00**

[52] **U.S. Cl.** **604/82**; 604/191; 604/43

[58] **Field of Search** 604/82, 191, 43, 604/275, 283, 289; 222/134, 135, 136, 137

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[57] **ABSTRACT**

This invention provides a medical device for delivering volumetric quantities of a first and a second biochemically reactive fluid comprising a first container having an opening, the first container being adapted to contain the first biochemically reactive fluid; a second container having a second fluid opening adjacent the first fluid opening, the second container being adapted to contain the second biochemically reactive fluid; a spray unit for separately atomizing the first and second biochemically reactive fluids into an aerosol with at least one energy source of a liquid energy, a mechanical energy, a vibration energy, and an electric energy; a fluid pressurizer for pressurizing the first and the second biochemically reactive fluids for delivery under pressure through the spray unit onto a surface; and wherein the first and second biochemically reactive fluids first mix on the surface.

7 Claims, 9 Drawing Sheets

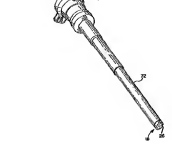

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FIG.1

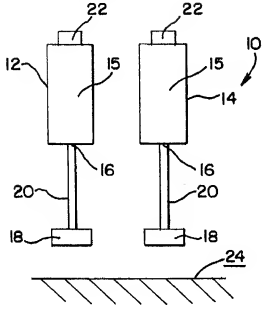

FIG.2

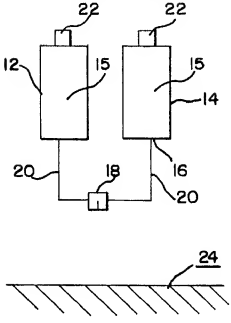

FIG.3

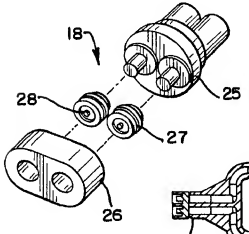

FIG.4

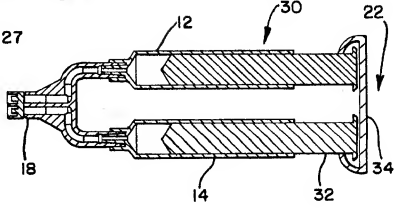

FIG.5

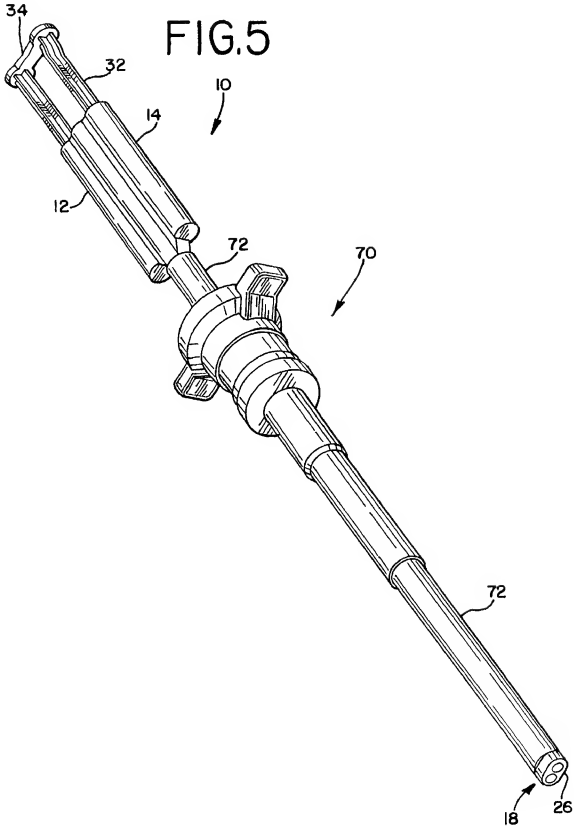

FIG.6A

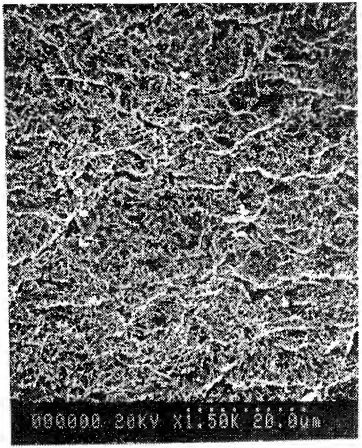

FIG.6B

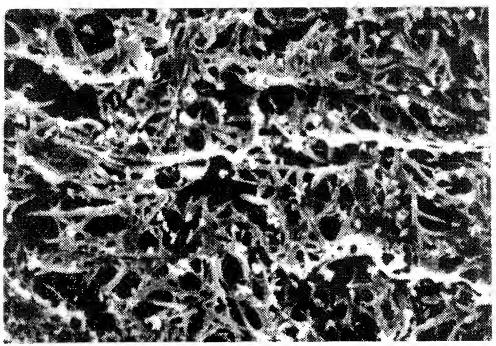

FIG.7A

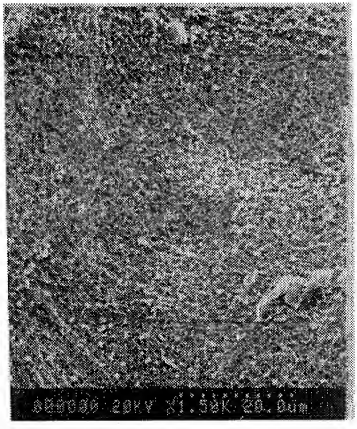

FIG.7B

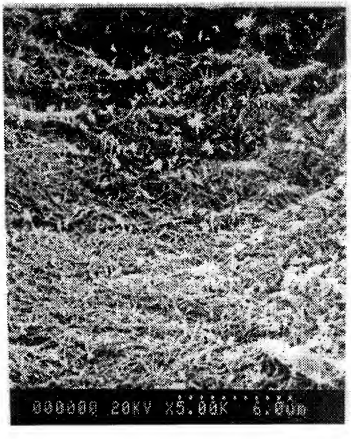

FIG.8A

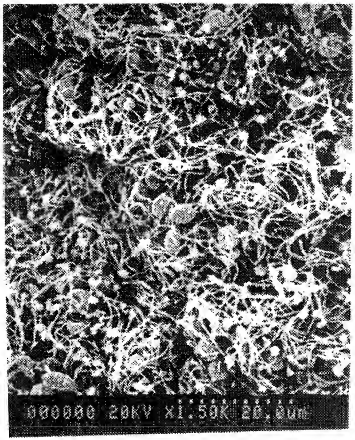

FIG.8B

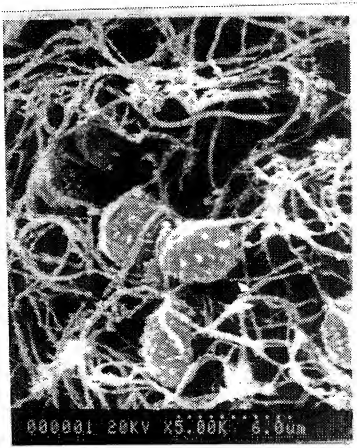

FIG.9A

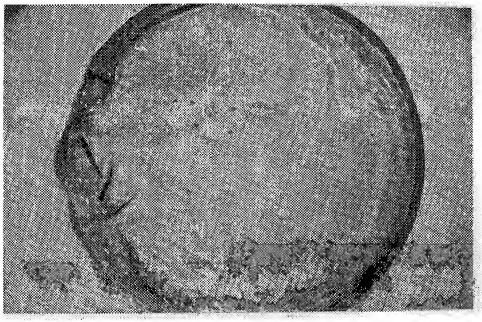

FIG.9B

FIG.10A

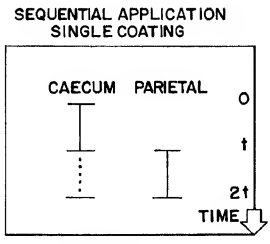

FIG.10B

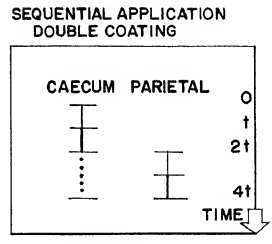

FIG.10C

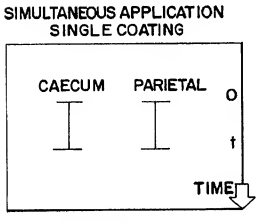

FIG.10D

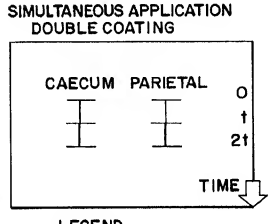

FIG. 11

CONTROL

.../.../... ADHESION (3) 100%

PARIETAL
CAECUM

SINGLE COATING

SEQUENTIAL APPLICATION: 5 MIN.
FG 4IU/-/FG4IU ADHESION(3) 100%
FG 100 IU/-/FG 100 IU ADHESION(3) 50%

PARIETAL
FG
CAECUM

DOUBLE COATING

SIMULTANEOUS APPLICATION : 5 MIN.
FG 4 IU & 100 IU/-/FG 4 IU & 100 IU ADHESION(1->3) 80%
FG 5 IU & 150 IU/-/FG 5 IU & 150 IU ADHESION (2) 25%

PARIETAL
FGs
CAECUM

FIBRIN FILM ALONE

---/FF 300 IU/--- NO ADHESION

PARIETAL
FF
CAECUM

SANDWICH METHOD

SIMULTANEOUS APPLICATION: 5 MIN.
FG 100 IU/FF 4 IU/FG 100 IU ADHESION (1- 2)60%

SEQUENTIAL APPLICATION : 7 MIN.
FG 100 IU/FF 4 IU/FG 100 IU NO ADHESION

SEQUENTIAL APPLICATION: 7 MIN.
FG 100 IU / FF 20 IU/FG 100 IU NO ADHESION

PARIETAL
FG
FF
CAECUM

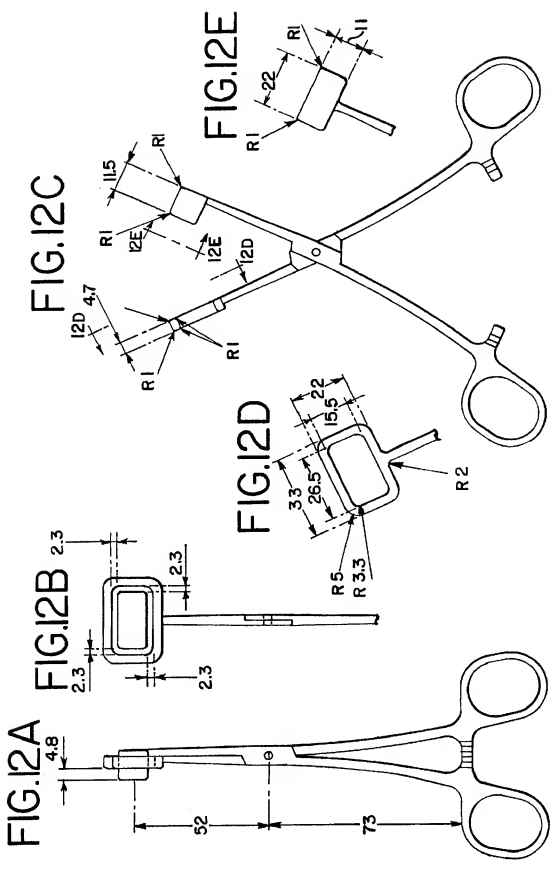
FIG.12A
4.8
52
73
FIG.12B
2.3
2.3
2.3
2.3
FIG.12C
12D
4.7
R1
R1
R1
12E
12E
12D
11.5
FIG.12D
22
15.5
33
26.5
R2
R5
R3.3
FIG.12E
R1
22
R1
11

FIBRIN DELIVERY DEVICE AND METHOD FOR FORMING FIBRIN ON A SURFACE

RELATED APPLICATIONS

This application is a continuation-in-part of PCT application number PCT/EP96/00160 filed Jan. 16, 1995 which claims priority from German patent application number 195 01 067.1 filed on Jan. 16, 1995.

TECHNICAL FIELD

This invention provides a medical device for simultaneously or sequentially delivering volumetric quantities of biochemically reactive fluids contained in separate containers and more particularly to a medical fluid delivery system for volumetrically delivering fibrinogen and thrombin to form fibrin on a surface.

BACKGROUND ART

One of the major problems in intra-abdominal surgery is the avoidance of post-operative adhesions. It is well-known that adhesions contribute to pain, immobility, retarded wound healing, and in particular to intestinal obstruction which even may be life-threatening. In the field of gynecological surgery, post-surgical adhesions involving female reproductive organs may result in infertility.

Each surgical procedure necessarily produces various forms of trauma where the abdominal cavity or other human cavity is opened for an inspection. Physiologically, the process of wound closure then starts when bleeding ceases upon formation of a hemostatic clot at the places where blood vessels are injured. The clot, at first comprising mainly platelets, is solidified by a fibrin network resulting from the activation of an enzyme cascade involving thrombin, factor XIII and calcium. Further steps on the way to the sealing of the wound are retraction of the hemostatic clot, invasion of various cell types including fibroblasts into the wound area and eventually the lysis of the fibrin network. Adhesions are thought to begin to form when the fibrin clot covering an injury comes into contact with an adjacent surface and the new connective tissue produced by the fibroblasts attach the two surfaces together.

The problems associated with adhesions often require a further operative procedure for removing/lysing the adhesions, called adhesiolysis, which, like the first operation, principally bears the risk of forming additional adhesions.

Accordingly, the prevention of adhesion formation is medically important. Among the different approaches for prevention of adhesion formation, one involves the use of materials as a physical or bio-mechanical barrier for the separation or isolation of traumatized tissues during the healing process. Both synthetic materials and natural materials have been used as a barrier to adhesion formation. Permanent, inert implants like Gore Tex® surgical membranes consisting of expanded polytetrafluoroethylene (PTFE) generally require a second operative procedure to remove them, while others such as surgical membranes of oxidized regenerated cellulose are biodegradable, but are thought to elicit an inflammatory response ultimately leading to adhesion formation (A. F. Haney and E. Doty, *Fertility and Sterility* 60, 550–558, 1993).

Fibrin sealants/glues are well-known in the art for use in hemostasis, tissue sealing and wound healing, and have been commercially available outside the United States for more than a decade. Fibrin glues have not been widely used for

anti-adhesion purposes. Further, the practice of changing the concentrations of thrombin and fibrinogen to achieve a fibrin film having a desired pore size is also not widely practiced.

Fibrin glues mimic the last step of the coagulation cascade and are usually commercialized as kits comprising two main components. The first component is a solution comprising fibrinogen and factor XIII, while the second component is a thrombin calcium solution. After mixing of components, the fibrinogen is proteolytically cleaved by thrombin and thus converted into fibrin monomers. Factor XIII is also cleaved by thrombin into its activated form (FXIIIa). FXIIIa cross links the fibrin monomers to form a three-dimensional network commonly called "Fibrin Gel."

Previous attempts to provide a thrombin and fibrinogen delivery device are known.

For example, one such device is disclosed in U.S. Pat. No. 4,978,336 Which discloses a dual syringe system. A device made by the assignee of the '336 Patent, Hemaedics, Inc., is sold under the tradename DUOFLO. Each syringe distal end is attached to a common manifold 14 having a mixing chamber. Fibrinogen and thrombin solutions are mixed in the manifold 14 prior to application to a wound or other surface. The manifold has a discharge tip for delivering the mixed solution onto a surface. The shortcoming of this device is the propensity for the tip to clog. This occurs when solid fibrin is formed upon brief interruptions in the application process. Such interruptions are common in normal medical procedures. The likelihood of this occurring increases as the thrombin concentration increases especially thrombin concentrations of greater than 20 IU/ml. The '336 Patent acknowledges the clogging problem and suggests solving the problem by replacing the clogged tip. (Col. 3, line 4–Col. 4, line 2). However, replacing clogged tips is impractical and unacceptable for minimally invasive surgeries where a cavity of an animal body is accessed through a small surgical opening.

Other techniques provide for applying beads of a solution of thrombin and calcium and a solution of fibrinogen and Factor XIII adjacent and in contact with one another on a surface. In this case, the thrombin and fibrinogen react primarily along interfacing surfaces while the remaining portions of the solutions are generally isolated from one another by the solid fibrin formed between them. Thus, there is inadequate mixing of the solutions to provide for a suitable fibrin film. Also, the unreacted fibrinogen is available to react with thrombin supplied by the body to promote the formation of adhesions.

U.S. Pat. No. 4,631,055 discloses another thrombin and fibrinogen delivery device having two syringes mounted in a holding frame 3 in parallel spaced relationship. A conical portion of a distal end each syringe is inserted into a connecting head. In one embodiment of the '055 patent, mixing of fluids contained in each syringe occurs inside the connecting head and in another embodiment the mixing of the fluids occurs outside the mixing head. The connecting head also includes a channel to supply medicinal gas under pressure. The medicinal gas contacts the fluids at a mouth of the connecting head and conveys the fluids contained in the syringes to a surface.

Product literature commenting on a dual syringe device for delivering fibrinogen and thrombin and sold by the Assignee of the '055 patent, reports that the device operates at gas pressures of about 30–65 psi. The momentum of the pressurized gas, especially when conveying entrained fluids, could possibly cause damage to tissue being treated by this device.

Finally, a device sold by Johnson & Johnson provided for applying a bovine thrombin and calcium chloride solution to a wound. In addition to possible issues raised by the use of bovine proteins, this procedure does not provide satisfactory hemostasis function in high blood flow situations. The thrombin is believed to be washed from the wound site by the flow of blood.

This invention overcomes these and other shortcomings in the prior art devices.

DISCLOSURE OF INVENTION

This invention provides a medical device for delivering volumetric quantities of a first and a second biochemically reactive fluid. The device comprises a first container having an opening, the first container is adapted to contain the first biochemically reactive fluid. A second container has a second fluid opening adjacent the first fluid opening; the second container is adapted to contain the second biochemically reactive fluid. A spray unit is in fluid communication with the first container and the second container, the spray unit being capable of separately atomizing the first and second biochemically reactive fluids into an aerosol with at least one energy source of a liquid energy, a mechanical energy, a vibration energy, and an electric energy. A fluid pressurizer is associated with the first and second containers for pressurizing the first and the second biochemically reactive fluids for delivery under pressure through the spray unit onto a surface. Wherein the first and second biochemically reactive fluids first mix on the surface. This device does not use any pressurized gas.

This invention also provides a method for delivering fibrin to a surface. The method comprises the steps of: (1) providing a liquid solution of fibrinogen; (2) providing a liquid solution of thrombin; (3) providing a spray unit in fluid communication with the fibrinogen and thrombin solutions, the spray unit being capable of separately atomizing the fibrinogen and the thrombin into an aerosol with at least one energy source of a liquid energy, a mechanical energy, a vibration energy, and an electric energy; (4) spraying the fibrinogen solution onto the surface with the spray unit; (5) spraying the thrombin solution separately from the fibrinogen onto the surface; and (6) mixing for the first time the fibrinogen with the thrombin on the surface to make fibrin.

This invention also provides a method for delivering fibrin to a surface within a cavity of a body of an animal to prevent the formation of adhesions. The method comprising the steps of: (1) providing a liquid solution of fibrinogen; (2) providing a liquid solution of thrombin; (3) providing a spray unit in fluid communication with the fibrinogen and thrombin solutions, the spray unit being capable of separately atomizing the fibrinogen and the thrombin fluids into an aerosol with at least one energy source of a liquid energy, a mechanical energy, a vibration energy, and an electric energy; (4) spraying the fibrinogen solution onto the surface with the spray unit; (5) spraying the thrombin solution separately from the fibrinogen solution onto the surface with the spray unit; and (6) mixing for the first time the fibrinogen with the thrombin on the surface to make a fibrin film capable of preventing the formation of adhesions proximate the fibrin film.

Preferably the medical devices of this invention operate at pressures that are generated by a hand-held device. This allows the present device to provide a cost effective and less complicated alternative to those medical devices that rely on pressurized gases as a means for atomizing fluids and conveying them to a surface to be treated.

It is contemplated that versions of the medical device shall be used in open-type surgeries such as laparotomic surgeries, topically and in minimally invasive surgeries such as laparoscopic surgeries. In open-type surgeries and minimally invasive surgeries, the present device may be used for hemostasis, tissue healing and antiadhesion purposes. In topical applications, the device may be used for treating burn patients during skin grafting procedures and to act as an antimicrobial agent.

The medical device is capable of simultaneously or sequentially delivering biochemically reactive fluids to a surface where they mix for the first time. In the sequential application, it is possible to repeat the procedure to incrementally form a fibrin film.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** is a schematic view of the device of this invention;

FIG. **2** is another embodiment of the device of this invention;

FIG. **3** is an assembly view in perspective of a spray unit;

FIG. **4** is a perspective view of the device of this invention for open-type surgeries such as laparotomic uses;

FIG. **5** is a perspective view of the device of the present invention for use in minimally invasive surgical techniques;

FIG. **6** is a scanning electron microscopy (SEM) of a fibrin film made of 3 IU of thrombin (mag. 1,5 K and 5.0 K). The pore size is about 3–4 μm.

FIG. **7** is a SEM of fibrin film made with 300 IU of thrombin (x 1,5 K, x 5.0 K). The pore size is about 0.2 μm;

FIG. **8** is a SEM observation of a blood clot obtained by mixing 100 μm of autologous blood with 100 μm of 20 mM $CaCl_2$ (x 1,5 K, x 5.0 K);

FIG. **9A** is a fibrin film in accordance with Example 1, containing methylene blue as a disinfectant;

FIG. **9B** is a fibrin film in accordance with Example 2;

FIG. **10** is a scheme showing the different types and modes of application using fibrin glues;

FIG. **11** is a scheme summarizing the different embodiments of the invention according to the different experimental approaches; and

FIG. **12** is a technical drawing showing different views of the clamp having been used in the course of the animal model.

BEST MODE FOR CARRYING OUT THE INVENTION

While the invention is susceptible of embodiment in many different forms, there is shown in the drawings and will herein be described in detail preferred embodiments of the invention with the understanding that the present disclosure is to be considered as an exemplification of the principles of the invention and is not intended to limit the broad aspect of the invention to the embodiments illustrated.

This invention provides a method and apparatus for delivering two biochemically reactive liquids, preferably fibrinogen and thrombin, to form fibrin. These biochemically reactive fluids may be delivered topically, in open-type surgeries such as laparotomic surgeries and in minimally invasive surgical techniques such as, for example, laparoscopically. While it is well known to use fibrin for hemostasis purposes, it has been found that fibrin can be used to prevent the formation of adhesions in cavities of the human body. It is possible to manipulate the concentrations of fibrinogen and thrombin, especially thrombin, to effect a

change in the characteristics of the resultant fibrin film. One such characteristic is the pore size of the film. By manipulating the concentrations of these fluids one can tailor the fibrin film that is best suited for its intended end use.

I. Fibrin Film

In a further aspect of the present invention, a combined use of a first fibrin glue acting as a hemostatic agent and of a second fibrin glue acting as a bio-mechanical barrier for the preparation of a medicament for the treatment of internal traumatic lesions is provided.

For the sake of convenience, the term "fibrin film" is used in the following to refer to a self-supporting sheet-like material of cross-linked fibrin. For the purposes of the invention, the fibrin film is composed of the same constituents as the fibrin glues on the market, i.e., mainly fibrinogen, thrombin, factor XIII and calcium.

Particularly advantageous is that the fibrin film does not considerably slip or move upon placement so that no fastening, like sutures, are required in the course of surgery. Due to its inherent mechanical properties, the fibrin film allows a tight cover over the traumatized tissue. Moreover, the fibrin film in terms of its composition and general structure is similar to the natural products formed upon injury in the human or animal body and, thus, they fulfill prime requirements an ideal surgical membrane would have, such as superior biocompatibility, biodegradability and bioresorbability. As a result and by marked contrast to barriers of inert materials like expanded PTFE, a bio-mechanical barrier made of fibrin disappears "by itself" after exerting its function so that no second surgical procedure for removal is required; in the animal model (rat) described below this usually occurs within ten days after the application of the fibrin film. Furthermore, it is appreciated that the fibrin film does not provoke any major side effects to the body.

In accordance with preferred embodiments of the invention, a self-supporting sheet-like material of cross-linked fibrin is provided, wherein said material has a regular pore size. The fibrin film of the invention has a dense, regular and homogenous structure within its entire volume as shown by Scanning Electron Microscopy (SEM). The pore size of the fibrin film is "regular" in the sense of varying only in a range of a few micrometers. It has been found that such a fibrin film is particularly effective in preventing adhesion formation.

On the other hand, according to experiments by the inventors, fibrin films having a more open or even irregular or non-homogenous structure including larger holes are not effective in this respect. Without being bound to a theory, this may be explained as follows. Such foam-, sponge- or fleece-like structures may allow the retention of blood set free during surgery and may further allow the ingrowth of fibroblasts, thus promoting endogenous fibrin formation accompanied by adhesion formation. Accordingly, such fibrin films, in particular in their dry state, may be useful in hemostasis to soak up the exudate of the injury. However, in anti-adhesion therapy, non-hemostatic fibrin films with non-adhesive properties are generally desired.

The fibrin film of the invention is insoluble in water and in the wet form may contain up to 92% by weight of water. Irrespective of being in hydrated (wet) or rehydrated state (e.g. after a previous drying step for storing), this fibrin film has a high mechanical strength, is by itself self-supporting and is yet soft. Thus, the fibrin film of the invention is easy to handle allows cutting, rolling and also suturing, if required. The self-supporting property of the fibrin film is reinforced by drying. The dry form of the fibrin film may be commercialized as part of a kit and is then to be rehydrated before use in surgery with appropriate solutions of e.g. water, calcium chloride, but also buffered solutions containing drugs.

Preferably, for the purposes of the present invention in certain embodiments, the pore size of the barrier material is below about 20 μm, more preferably below 5 μm, and most preferably below about 1 μm, to prevent fibroblasts from intruding or penetrating. As noted above, in the course of normal wound closure, fibroblasts migrate into the fibrin clot network and the developing granulation tissue, where they produce i.e., collagen and thus contribute to the ultimate formation of a scar tissue. In order to avoid that the substances produced by the fibroblasts contribute to glue, an injured surface and an adjacent surface or two injured surfaces together, the inventors propose to isolate or separate the injured surface(s) by using fibrin barrier material having a pore size, preferably a "regular" pore size in the sense of the present invention, of below 20 μm, preferably below 5 μm, preferably of below 4 μm, preferably of below 3 μm, preferably of below 2 μm and most preferably of below 1 μm or any combination of ranges between these sizes inclusive of the endpoints. In fact, experiments by the inventors described below demonstrate that by using such barriers the formation of adhesions can be prevented completely.

Usually, fibrin film in accordance with the invention is made of a single layer which, for the purpose of preventing adhesion formation, has a closed structure, but may also have an open structure for other applications. Moreover, the inventors propose a fibrin film comprising two or more layers. At least one layer, either as an outer layer or an intermediate layer, should have a closed structure ensuring the rigidity and/or the barrier function of the multi-layered fibrin film, whereas other layers having a open structure may work as a drug delivery system.

In preferred embodiments, the thickness of the fibrin barrier material is at least 20 μm when the barrier is in the wet state. Preferably the thickness is about 20–2000 μm, and most preferably up to 5000 μm, although it is believed that even material with a thickness of less than 20 μm may be suitable for the purposes of the invention.

It is well known that thrombin acts as a protease which will cleave fibrino peptide A and B from the fibrinogen molecule and convert it into fibrin. It is desirable that all of the fibrinogen be converted into fibrin, as residual amounts of fibrinogen may lead to adhesion formation upon reacting with thrombin provided by the body. The rate of the conversion of fibrinogen into fibrin increases as the concentration of thrombin increases, provided that there is a sufficient quantity of fibrinogen present. Preferably, thrombin is added at a ratio of 7 parts by weight for every 1 part by weight of fibrinogen, and more preferably within the range of 6 to 1 a more preferably within the range of 4 to 1 and most preferably 1 to 1. The rate of the conversion determines the pore size of the resultant fibrin film. The faster the conversion to fibrin the smaller the pore size of the resulting fibrin film. Using a thrombin solution having a concentration of about 3 IU/ml, approximately that found in the human body, produces a fibrin film with a relatively large pore size. The large pore size fibrin film is suitable for hemostasis and wound healing. Accordingly, in still further embodiments of the present invention, the fibrin film further comprises less than 5% by weight of fibrinogen, preferably less than 4% by weight of fibrinogen, preferably less than 3% by weight of fibrinogen, preferably less than 2% by weight of fibrinogen, and most preferably less than 1% by weight of fibrinogen, in terms of the total dry weight of the fibrinogen plus fibrin each time.

Generally speaking, the lower the amount of residual fibrinogen, the better the non-adhesive properties of the fibrin film, since fibrinogen in vivo may promote fibrin formation and thus adhesion formation. For the purpose of determining the fibrin and the fibrinogen content of the fibrin film, the methods of SDS-Page (SDS-Gelelectrophoresis) may be used.

It is preferred in certain embodiments that the fibrin film further comprises one or more disinfectants, preferably methylene blue, and/or one or more drugs selected from antibiotics, fibrinolytic agents and biological response modifiers, in particular cytokines and wound repair promoters, preferably in an amount up to 1% by weight in terms of the total dry weight of fibrin plus fibrinogen. Examples of fibrinolytic agents include t-PA, μ-PA, streptokinase, staphylokinase, plasminogen and the like. These compounds promote fibrinolysis and thus can be used for controlling the rate of the degradation of the fibrin film in vivo. The term "biological response modifiers" is meant to refer to substances which are involved in modifying a biological response, such as wound repair, in a manner which enhances the desired therapeutic effect. Examples include cytokines, growth factors and the like. Due to its intrinsic mechanical properties, the fibrin film of the invention does not require any additional cross-linking agent which may exert any toxical effects to the human or animal body.

II. Biochemically Reactive Solutions

The components of the fibrinogen and thrombin can be prepared from plasma by conventional precipitation and purification steps. When the patient to be treated is a human being, human plasma will be preferred. The source of the plasma may be either pooled donor blood and single donor blood obtainable from blood centers, respectively. Care should be taken that state of the art controls are performed to detect viral contamination. During the process of manufacturing, the products may be sterilized by standard techniques as well. In order to avoid any risk of contamination, the components could be prepared from pre-operative autologous blood donation. It will be understood that the components of the first or the second solution or their functional analogues may also be prepared by using the methods of molecular genetics.

Conveniently, in the light of the present disclosure, commercially available two-component fibrin glue kits may be used for the preparation of the fibrin film of the present invention. The required constituents are usually contained in the kits in the form of lyophilized concentrates and have to be reconstituted as per the technical data sheet provided with the respective kit. The desired thrombin concentration is prepared by diluting an aliquot of the reconstituted thrombin solution with sterile calcium chloride solution, preferably 20 mM or 40 mM calcium chloride.

The inventors propose that the fibrin film of the invention may also be obtained from one vial containing all the required components, where the catalytic agents for the fibrinogen-fibrin conversion and the cross-linking of soluble fibrin, respectively, are inactivated and the polymerization is only started by induction through a change in pH, ionic strength, light and the like after the content of said vial had been applied to the solid support. By way of example, photo-sensitive inhibitors of thrombin and thrombin-like molecules could be used for this purpose. The fibrin film of the invention may also be prepared in accordance with Copley and Luchini, (*Life Sciences* 3, 1293–1305, 1964) and Sasaki et al. (*Science* 152, 1069–1071, 1966) by starting from soluble fibrinogen-fibrin monomer complexes precipitated in the cold, redissolved at high temperature and which are then cross-linked with F XIII and calcium.

In accordance with the invention, the first solution preferably contains fibrinogen and factor XIII (10–40 IU/ml). The concentration of fibrinogen is expressed as the total protein concentration (preferably from about 15–140 mg/l and more preferably 30–110 mg/ml) and the percentage of clottable protein comprised therein. It is also preferred that the fibrinogen solution have a viscosity that allows the solution to be sprayed and preferably sprayed using pressures generated using a hand-operated syringe. The fibrinogen solution should have a viscosity of less than 20 centipoise, more preferably less than 10 centipoise, and most preferably from 1–5 centipoise or any combination or subcombination of ranges therein.

The inventors prefer the percentage of clottable protein to be at least 80% and preferably equal to or greater than 90%. Of course, those of skill in the art will recognize that a variety of other constituents may be included in the first solution, for example albumin, plasminogen and tensides. The thrombin solution preferably comprises 3–10,000 IU/ml, even more preferably 200–500 IU/ml, and most preferably 400–500 IU/ml or any combination or subcombination of ranges therein (depending on the desired biophysical parameters of the material to be obtained) and calcium in a concentration of up to 45 mM. For simplification, the thrombin concentration normally given in IU/ml, will in the following frequently be indicated in IU, in particular in the Tables.

III. Delivery Device

FIG. **1** shows a schematic representation of a device **10** having a first container **12** and a second container **14**. Each of the containers have a fluid channel **15** and a fluid opening **16**. The opening **16** of each of the first and second containers **12** and **14** are associated with a spray unit **18**. The spray unit **18** may be directly attached to the first and second containers **12** and **14** or be connected by other means such as a flexible medical tubing **20**. A pressurizer **22** is associated with each of the first and second containers **12** and **14** for pressurizing fluids that will be contained therein for delivery to a surface **24**. It is also possible to have a single pressurizer for both containers.

FIG. **2** shows a delivery device having a single spray unit **18**. FIG. **3** shows that the spray unit **18** is an assembly of several parts typically having an input piece **25**, and an output piece **26** together sandwiching two mechanical break-up units (MBU) **27**. Preferably the pieces **25** and **26** snap fit together for ease of assembly.

The MBU is what is known in the art as a jet swirl atomizer. The MBU's **27** have an inner surface having three converging tangential channels that define a fluid path that rotates in a clockwise direction as viewed from an inside surface of the MBU looking out. The channels direct the incoming fluid to a spin chamber to generate angular momentum in the fluid. The spinning fluid exits the MBU through a port **28** to form an aerosol.

It may be desirable to have one MBU having channels that follow a clockwise path and another adjacent MBU that has channels that travel counterclockwise. It is also possible that the MBU's **27** have from 2–4 channels per spray unit or more.

Several presently preferred MBUs are available from Seaquist Dispensing of Cary, Ill. under the product designations CS-5512, CS-5501, and CS-5503.

The device shown in FIG. **1** would have a single MBU per spray unit **18**. The device shown in FIG. **2** would have the spray unit shown in FIG. **3** having two MBUs **27** per spray

unit **18**. Of course it is possible to incorporate more than two MBUs per spray unit **18**.

Generally, it is possible to use several different energy types to form an aerosol from the biochemically reactive fluids. The preferred energy types are those selected from the group consisting of liquid energy, mechanical energy, vibration energy, and electric energy. This group excludes gas energy which is employed in U.S. Pat. No. 4,631,055 as the mechanisms necessary to generate the gas energy may be expensive and because the momentum of the gas energy stream that atomizes the fluids may be incompatible with and cause damage to certain delicate human tissues. Mechanisms capable of generating these energies and separately atomizing fluids may be referred to in the claims as a means for separately atomizing first and second fluid streams or the like.

Atomizers that use liquid energy are the preferred devices for generating an aerosol spray and include swirl atomizers and most preferably jet-swirl atomizers such as the MBUs **27** described above. An example of an atomizer employing mechanical energy includes rotary atomizers such as impellers or pumps. An example of devices employing vibration energy include acoustic and ultrasonic devices. An example of devices employing electric energy to create an aerosol spray include electrostatic devices. These are all well recognized energy sources for atomizing liquids as set forth in (L. Bayvel and Z. Orzechowski, *Liquid Atomization, pg.* 2).

These atomizing devices could also include a piezoelectric crystal that meters out small droplets of fluid based upon a cycle time of the piezoelectric crystal.

Preferably, the spray unit **18** has a diameter of 10 mm or less so that it may be passed through standard trocar devices which typically have diameters of from about 10 mm–12 mm and preferably 5 mm. Trocars are used to access internal cavities of an animal body during minimally invasive surgeries. The input device **25** shown in FIG. **3** has an external diameter of less than about 10 mm–12 mm.

It is also important that there be proper spacing between the two MBUs **27** to achieve mixing of the two biochemically reactive fluids on the surface **24**. It is also important that the MBUs **27** have sufficient spacing to prevent mixing at the discharge port **28** of each MBU. Such mixing would cause fibrin to form at the exit port **28** thereby clogging the device **10**. This invention further contemplates having a barrier wall (not shown) separating each MBU to prevent such mixing.

As shown in FIG. **4**, the containers **12** and **14** are preferably syringes and are attached together or are integral with one another to define a single unit **30**. The syringes should be of a size commonly available and have volumes from about 1–20 cc and most preferably 10 cc. It is also preferable that the containers **12** and **14** have equal volumes.

The pressurizer **22** in this embodiment is a dual plunger having two horizontally spaced plungers **32** mechanically coupled at one end by a crossbar **34**. FIG. **4** could also be modified such that distal ends of the containers **12** and **14** are dimensioned to fit directly into rear inlet ports **35** on the input device **25** (FIG. **3**).

It should be understood that in place of the syringes, this invention contemplates using pipettes or other devices that are capable of dispensing accurate and determined volumes of liquid. One presently preferred pipette is a repeatable pipette sold by Eppendorf. The pressurizer could also be other devices capable of generating fluid pressure within a container such as a pump. The invention also contemplates using more than two containers to deliver additional fluids to the surface **24**.

IV. Delivery Device for Minimally Invasive Surgery

FIG. **5** shows the medical device **10** adapted for use in minimally invasive surgical applications. Device **10** has medical tubings **20** which extend from the first and second containers **12** and **14** through a sleeve **72**. The sleeve **72** extends through a trocar **70** which is inserted into a surgical opening of an animal body to provide access to a cavity of the animal. In this fashion the spray unit **18** may be directed into the animal cavity to treat a wound therein.

This invention contemplates providing an articulatable joint (not shown) at a distal end of the device **10** which may be controlled by medical personnel outside the animal cavity to position the spray unit **18** to face a wound or surface to be treated with the device **10**.

V. Method of Using Device

The medical device **10** of this invention may be used topically, in open-type surgeries (for example, laparotomic surgeries) or minimally invasive surgeries (for example, laparoscopic surgeries). Of course, there are other types of open-type surgeries and minimally invasive as will be appreciated by one of ordinary skill in the art. By mixing the fibrinogen and thrombin outside the device **10**, the device may deliver high concentration thrombin solutions without clogging. The medical device **10** may be used to form fibrin films outside the human body using low thrombin concentrations and high thrombin concentrations.

1. Formation of Fibrin Outside Body Using Low Thrombin Concentration

The present invention is also concerned with processes of preparing a self-supporting sheet-like material of cross-linked fibrin.

Accordingly, in certain embodiments of the invention, a process of preparing a self-supporting sheet-like material of cross-linked fibrin is provided, which process comprises the steps of:

(a) simultaneously mixing a stream of a first, fibrinogen-containing solution with a stream of a second, thrombin-containing solution;

(b) applying the obtained mixture to a solid support; and

(c) incubating the mixture to form the desired material.

In order to obtain a mixture as homogenous as possible (and thus a homogenous final product) in step (a), a stream of a first, fibrinogen-containing solution is simultaneously mixed with a stream of a second, thrombin-containing solution. The first and/or the second solution may further comprise disinfectants and/or drugs selected from antibiotics, fibrinolytic agents and biological response modifiers, in particular cytokines and wound repair promoters. Preferably, equal volumes of the first and the second solution are mixed. In case the different volumes of the first and the second solution should be simultaneously mixed, it will be known in the art which measures have to be taken in order to ensure that a homogenous mixture is obtained.

Using the delivery device described above, the resulting mixture is spread over the surface of a solid support, for example a petri dish and the like, which is tilted to cover the entire surface as far as possible before the formation of the three-dimensional fibrin network starts. Using this preparation mode, fibrin films made with low concentrations of thrombin can easily be obtained. With higher concentrations of thrombin, a faster clotting time and thus a rapidly increasing viscosity of the mixture are observed as main limitations for the mixing procedure described above. Accordingly, for higher thrombin concentrations, care has to be taken that the mixture formed in accordance with step (a) is uniformly distributed over the surface of the solid support from the beginning, so as to yield a homogenous final product in step (c).

Step (c) preferably requires that the mixture applied to the solid support is allowed to set completely, i.e., a conversion of fibrinogen to fibrin as complete as possible is obtained. Preferably, completion of the conversion of fibrinogen to fibrin is achieved by incubation of the solid support at the physiological temperature, i.e., 37° C., for 1–200 minutes. It will be appreciated that the incubation may also be extended up to 24 hours and more. In this respect it is noted that the invention shall also cover those products, where the fibrinogen to fibrin conversion has not reached completion.

2. Formation of Fibrin Outside Body Using High Thrombin Concentration

As an alternative, in particular for the purpose of preparing a fibrin film with a higher concentration of thrombin, the inventors propose a process comprising the steps of:

(a) applying a first, aqueous, fibrinogen-containing solution onto a solid support;

(b) removing the water until dryness while forming a sheet-like fibrinogen material;

(c) applying to the sheet-like fibrinogen material a second, thrombin-containing solution; and

(d) incubating to form the desired material.

Whereas the specific steps of this process differ from those of the previously described process for the preparation of a fibrin film, the same first and second solutions may be used. In a preferred embodiment of said process, equal volumes of the first and the second solution are used in steps (a) and (c).

In order to obtain a final product having a regular thickness and a homogenous structure the first, aqueous, fibrinogen-containing solution should be uniformly distributed over the entire solid support. Step (b) requires that the solvent of the first solution, i.e. water, is removed until dryness in order to obtain a sheet-like fibrinogen material. Preferably, removal of water is performed by air drying, freeze drying, or drying under increased temperature and/or reduced pressure. The obtained sheet-like fibrinogen material has microcavities as shown by SEM and, thus, a high absorptive capacity for fluids. Said material is converted into a self-supporting sheet-like material of cross-linked fibrin upon rehydration by means of the addition of a second, thrombin-containing solution, which optionally comprises disinfectants and/or drugs like antibiotics, fibrinolytic agents, biological response modifiers and the like.

According to step (d), the solid support and, thus, the intermediate product of step (c), is incubated to form the self-supporting sheet-like material of cross-linked fibrin, i.e. the final product. Preferably, step (d) comprises incubating the solid support at 37° C. for about 20 minutes (with material of low thickness) to about 200 minutes (with material of high thickness) to complete the conversion of fibrinogen to fibrin. It will be appreciated that the incubation to form the final product may be extended to up to 24 hours and more.

It has been found that, with this process, the thickness of the fibrin film is independent of the concentration of the thrombin solution used. The fibrin film obtained has a high mechanical strength and can be cut, bent, rolled and sutured, and has a regular surface. In terms of the process, a particular advantage resides in that it is not dependent on the clotting time. That is, no premature clotting may occur, since the first and the second solution are separately applied to the solid support.

It is, of course, recognized that the preliminary process steps of the two processes described above are preferred laboratory procedures that might be readily replaced with other procedures of equivalent effect.

3. Open-type surgeries and topical application

In open-type surgeries, and in topical applications, the spray unit **18** of the device **10** shown in FIG. **4**, is positioned facing a surface such as a wound or a surface proximate the wound. The first and second containers **12** and **14** are consecutively or simultaneously pressurized to deliver to the surface the thrombin and the fibrinogen for mixing on the surface. Preferably the thrombin has a concentration from 3–10,000 IU/ml.

4. Minimally Invasive Surgeries

In minimally invasive surgical applications, the invention provides a method for delivering fibrin to a surface to be treated of an animal, or to isolate one surface from another, to prevent the formation of adhesions. The method comprises the steps of: (1) providing a liquid solution of fibrinogen; (2) providing a liquid solution of thrombin having a concentration from 3–10,000 IU/ml and more preferably from 200–500 IU/ml; (3) providing a spray unit in fluid communication with the fibrinogen and thrombin solutions, the spray unit being capable of separately atomizing the fibrinogen and the thrombin into an aerosol with an energy selected from the group consisting of liquid energy, mechanical energy, vibration energy, and electric energy; (4) spraying the fibrinogen solution onto the surface with the spray unit; (5) spraying the thrombin solution separately from the fibrinogen solution onto the surface; and (6) mixing for the first time the fibrinogen with the thrombin on the surface to make a fibrin film, in situ. The film is capable of preventing the formation of adhesions proximate the fibrin film. The trocar **70** and sheathe **72**, described above and shown in FIG. **5**, may be used to provide access to the animal cavity.

VI. Use of Fibrin Film

Generally speaking, the main determinants in influencing the fibrin network structure and its biological and biophysical characteristics include the concentrations of thrombin, fibrinogen and factor XIII, and, of course, the temperature at which the polymerization is performed. The fibrinogen concentration and, in a large measure, the clottable protein concentration is proportional to the tensile strength, while the concentration of factor XIIIa which covalently cross-links the fibrin monomers influences the elasticity of the fibrin network.

However, the thrombin concentration plays a key function for controlling fibrin network formation. That is, the biopolymer structure is inversely related to the thrombin concentration, while keeping the same regular and uniform structure at each concentration of thrombin. At low concentrations of thrombin, there is a slow fibrinogen conversion associated with a slow fiber growth, thus leading to the formation of a fibrin structure with thick fibers and large pore size (typically greater than 1 μm and most preferably greater than 20 μm). In other words, a low thrombin concentration leads to a long clotting time and larger pores. On the other hand, high concentrations of thrombin result in shorter clotting times producing a tight material with thinner fibrin fibers and smaller pore size (most preferably <1 μm and more preferably less than 0.2 μm). This effect can be demonstrated by using standard scanning electron microscopy (SEM). While FIG. **6** shows the network of a fibrin film developed with a low thrombin concentration (3 IU) having a pore size of greater than 1 μm (about 3–4 μm), FIG. **7** shows a fibrin film made with a high thrombin concentration (300 IU) having a pore size below 1 μm (about 0.2 μm). For comparison, FIG. **8** shows a representative human fibrin clot structure. In the human hemostatic clot, the presence of cells drastically opens the three-dimensional structure of the

network. Such an opened and irregular structure is physiologically favorable to fibroblast migration into the fibrin clot network during the normal wound healing process. It is apparent from the figures that by varying the thrombin concentration, fibrin networks with low or high pore size are obtainable. For the use of the fibrin material as a bio-mechanical barrier in accordance with the present invention, the thrombin concentration is preferably adjusted to obtain a fibrin network structure with a pore size excluding fibroblast penetration. The fibrin material produced in accordance with the invention may be examined by standard SEM and further be tested in the animal model described herein.

In view of these findings, the inventors propose that a fibrin film with a highly ordered structure having a "low pore size" is useful as a biomechanical barrier to avoid contacts between adjacent injured surfaces. Additionally, it is proposed to use a fibrin film with a highly ordered structure having "relatively large pores" as a matrix for cells and molecules for the achievement of hemostasis and wound repair.

This is all the more important as the inventors using the animal model described below, have found that the development of adhesion requires blood, peritoneal trauma, and approximation/contact of injured surfaces.

Under consideration of these three factors, in certain embodiments hemostasis and wound repair is addressed by applying a single layer of fibrin glue to the injury site(s), while the separation/isolation of the injured surface(s) is achieved by using a bio-mechanical fibrin barrier, either applied as a second layer on top of the first layer of the fibrin glue, or simply as a self-supporting sheet placed between the adjacent injury sites or between the site of the injury and the adjacent uninjured tissues. The inventors have discovered that an important parameter to be taken into account in using such a combination of a hemostatic agent/wound repair promoter and a bio-mechanical barrier is the time required for complete conversion of fibrinogen to fibrin. Specifically, it has been found that the layer of the fibrin glue and respectively the last layer, if more than one layer is applied to an injured surface, should be allowed to set until the conversion of fibrinogen to fibrin is complete. By way of example, when fibrin glue is applied simultaneously to two injured surfaces such as caecum and peritoneal wall in order to form a single layer each time, and the surfaces come into contact with each other before the fibrinogen-fibrin conversion is complete, it may occur that these surfaces are glued together, i.e., that adhesions are formed.

This means that the surface state of the different interfaces and their relationships are very important in the prevention of adhesions. As a general guidance, the inventors propose to allow undisturbed setting after application of the respective last external layer of fibrin glue until the conversion of fibrinogen to fibrin is complete. This does not apply to the fibrin film of the invention, since this is allowed to set completely in vitro before application. Of course, although detailed experimental protocols are described hereinafter, those of skill in the art will appreciate that the specific time requirements may vary depending on the particular patient, the type of injury and the handling, and is thus apparently also a matter of clinical experience. However, in vitro methods are known in the art for monitoring the fibrinogen-fibrin conversion. By way of example this can be followed by monitoring turbidity which is the measure of the optical density of fibrin networks developed in a cuvette with a path of one centimeter at 800 nm (cf. G. A. Shah et al., *Thrombosis Research* 40, 818–188, 1985). In accordance with this method it is possible to determine in vitro the time required for complete fibrin formation at a given thrombin concentration. This provides an estimate of the minimum time required for complete setting after application of the last external layer(s). It is believed that, based on the present disclosure, one of ordinary skill in the art could define a protocol for use of a dedicated fibrin glue, its mode and type of application, so that the requirements for surgeons with respect to the timing and the technical devices are met.

In accordance with the general guidelines described above, a preferred embodiment called "double coating" comprises the application of a first fibrin glue with a low concentration of thrombin to work as hemostatic agent and/or tissue repair promoter, and of a second fibrin glue with a high concentration of thrombin playing the role of a bio-mechanical barrier which entirely covers the injury and the first coating formed upon application of the first fibrin glue. Preferably the first fibrin glue has been made by mixing of the above-described fibrinogen-containing solution with an equal volume or a thrombin-containing solution comprising less than 1000 IU thrombin, preferably less than 150 IU. The fibrin glue has been preferably made by mixing said fibrinogen-containing solution with an equal volume of a thrombin-containing solution of at least 50 IU thrombin, preferably of at least 150 IU thrombin, and most preferably of least 300 IU thrombin. Of course, it will also be possible to apply more than two layers as long as the last layer plays the role as a biomechanical barrier preventing fibroblast proliferation between the covered lesion and the adjacent surfaces.

In another preferred embodiment of the invention called "sandwich method," a fibrin glue layer covering the injured surface(s) is used as hemostatic agent and wound repair promoter, while a fibrin film, in i.e., a self-supporting sheet-like material of cross-linked fibrin being placed between the injured surface and an adjacent uninjured surface, or between two injured surfaces, acts as a bio-mechanical barrier. The fibrin glue is preferably produced by mixing of a first, fibrinogen-containing solution with an equal volume of a thrombin-containing solution comprising preferably 1–300 IU/ml thrombin, preferably at least 20 IU/ml thrombin and most preferably at least 100 IU/ml thrombin. The fibrin film is made of at least 4 IU/ml thrombin, preferably of at least 20 IU/ml thrombin, and most preferably of at least 300 IU/ml thrombin. It will, of course, be recognized that the fibrin film can also be used in combination with a double coating as described above.

The above-described embodiments may be used either alone or in combination with other embodiments.

VII. Hemostasis and Antiadhesion Purposes

The device 10 and the methods described above for using the device, are effective for both hemostasis and antiadhesion purposes. For hemostasis, it is preferable to apply a thrombin solution having a thrombin concentration of less than 400 IU/ml and more preferably from 1–10 IU/ml. The application of both fibrinogen and thrombin are even effective in high blood flow applications.

For antiadhesion purposes, it is preferable to use a thrombin concentration of from about 10–10,000 IU/ml and more preferably from 200–500 IU/ml. The biochemically reactive fluids are applied to a surface such as a wound or neighboring tissue to form a fibrin film capable of isolating tissues from healing tissues.

VIII. Sequential and Simultaneous Pressurization

As stated above, it is possible to deliver the thrombin and the fibrinogen sequentially or simultaneously. In simultaneous applications, the fibrinogen and thrombin are applied in amounts sufficient to have the desired function of hemostasis or of forming a fibrin film of sufficient thickness to act as a barrier.

In sequential applications, the fibrinogen and thrombin may be applied in either order but preferably the thrombin is sprayed on first followed by the fibrinogen. To form a fibrin film for antiadhesion purposes having a uniform and regular surface, it is preferred that the fibrin film be formed incrementally. This incremental approach requires spraying small volumes, such as 0.3 ml or any volume that may be somewhat accurately measured using a syringe, of each of the biochemically reactive fluids onto the surface and repeating this process until the fibrin film has a desired thickness. This process may have to be repeated from about 1–5 times to form a fibrin film having a thickness of say about 500 μm. This incremental process overcomes the natural tendency for the later sprayed fluid to displace the earlier sprayed fluid creating an irregular surface in the fibrin film. By spraying small fluid volumes the fluid displacement is minimized.

IX. Materials and Methods

1.1 Animals

For these studies, female Wistar rats weighing 180–200 g each were used. Before surgery the animals were kept in groups in cages and after surgery until necropsy they were kept alone. The strain designation is ICO.WI/(IOPS, CPB). The experiments were performed following the guidelines of the "Législation et réglementation relative à l'expérimentation animale."

1.2 Materials

For these experiments, Baxter Fibrin Sealants kits lot # 26302001AA were used. While these kits are not on the market yet, in the light of the present disclosure any standard two-component fibrin sealant kit may be used for the purposes of the invention.

Calcium Chloride 20 mM from Nycomed lot# 931343 Petri dish (9 cm diameter) from Nunc (cell culture quality) were used.

1.3 Surgical and Anaesthetic Equipment

The surgical equipment included: PVC plate (surgery table) 30×30 cm; needle holders CRILE-WOOD, 14 cm; forceps; Wangesteen, 15 cm; standard anatomic forceps, 13 cm; dissecting scissors, Metzenbaum, 14 cm; home-made clamp (for fixing a tissue surface of 2 cm^2); lamp, Well-Lynch; scalpels SWANN-MORTON disposable # 24; surgical suture USP Dermalon non-absorbable, Dermalon 2.0 needle CE-4, Dermalon 4.0 needle CE-6; gauze swabs: layer 12, 10×10 cm Molnlycke. Diethyl ether was used as anaesthetic.

The home-made clamp shown in FIG. **12** serves as a tool to standardize the injuries inflicted on the surface of the parietal wall in terms of their position, area and depth. The clamp consists of two mobile parts. The "male" part has rounded edges and embraces an area of 2 cm^2 corresponding to the surface of the smallest caecum found in a rat. When the clamp is closed, there is a gap of 2 mm between the male and the female part, which gap is sufficient to keep the respective tissue (muscle layer, skin) immobile without any shearing or cutting. The mechanical tension induced by the clamp is necessary to allow easy separation of the first muscle layer from the second one when the clamped surface is incised with a scalpel. The tension allows better control of the depth of incision.

1.4 Disinfection and Biological Waste

Surgery equipment was disinfected and washed with 15–20 g/l Mucocit-R (Merz) as per the technical data sheet. For all materials as well as the dead animal carcasses, usual measures for waste disposal were taken.

1.5 Animal Surgery

The surgical procedure as described below, using the above-described clamp, allows production of adhesions of the same type in control experiments with an incidence of 100 percent.

The skin was cut following a 4 cm line joining the xyphoid and urinary aperture for a rat weighing 180–200 g with a pair of Metzenbaum scissors.

The skin edges were lifted and carefully dissected from the muscle wall on each side of the linca alba.

The abdominal muscle was incised along the linea alba over the 4 cm line as described above.

The caecum was gently removed and laid on gauze swab avoiding, at any time, contact with the latex gloves and damage by instruments.

The caecum was abraded with gauze on its upper side until only punctuate bleeding appeared.

The caecum was returned to the peritoneal cavity if no treatment had to be performed. If a treatment was carried out, the product to be tested was applied on the caecum, which was then gently returned to the peritoneal cavity.

In the next step, the home-made clamp described above was used which had been designed to standardize the injury inflicted on the parietal wall in terms of area, position and depth, thus duced. Then the clamp was turned inside out to expose the parietal wall.

The exposed serosal surface was incised through the first muscular layer with 2×10 crossed incisions of constant depth.

After this step, the incised surface was replaced on the abraded caecum if no treatment had to be performed. If a treatment was performed, the product was applied on the incised surface and then carefully replaced on the abraded caecum such that caecum and parietal wall were separated by the product.

At that time, care was taken to avoid any movement that could induce distension or stretching, especially to the parietal caecum side.

The muscle was sutured with non-absorbable dermalon 2.0 and the skin closed with dermalon 4.0.

The animal identification was performed by using an electronic tag (ZETES Electronic Inc.) defined by an univocal key of 12 alpha-numeric digits.

Then the animals were allowed to recover in the laboratory.

1.6 Sham Control

A "Sham" was performed by following all the steps of the surgical procedure, but without inflicting incisions or abrasions. Surgery timing was rigorously observed.

1.7 Animal Sacrifice

The animals were sacrificed after 10 days. The post-mortem viscera studies were made through a U-shaped abdominal incision started at the liver level. Adhesions, present in the peritoneal cavity, were evaluated by two independent investigators.

1.8 Adhesion Classification

The adhesions were recorded according to their nature and tensile characteristics.

1.8.1 Nature of the Adhesion

The adhesions were classified in a table depending on the organs involved in the adhesion process. The adhesions involving the sutures were recorded, but will not be listed as adhesions hereinafter.

The main adhesion type observed and classified are:

caecum/parietal

visceral/visceral

fat/parietal

fat/visceral

fat/fat

The name of the fat or viscera in relationship with the "nature of the adhesion" will also be mentioned in parentheses.

e.g.: grade 2 adhesion between the fat of the uterus and the parietal wall will be reported as follows:

fat/par

(Ut) (2)

Ovary (O), Colon (Co), Ileum (Il), Bladder (Bl), and Omentum (Om) may also be involved in the adhesion process.

1.8.2 Tensile Characteristics

The adhesion was pealed off and evaluated according to the macromorphological adhesion grading scale (MAS) as follows:

0: no adhesion

1: filmy (mild)

2: adhesive bands (medium)

3: extensive adhesion formation (severe)

The adhesion tensile value was recorded in the appropriate column of the respective table with the type of organ involved in the adhesion process, as described above.

1.9 Type of Application

Two different types of application were performed with fibrin glue (FG): a "single coating" (one layer) or a "double coating" (two layers).

1.9.1 Single Coating

A fibrin glue with a defined thrombin concentration was used as an hemostatic agent and tissue repair promoter, respectively.

Fibrin glue was applied as a "single coating" on the caecum and the parietal wall as described in paragraph 1.5.

1.9.2 Double Coating

Two fibrin glues, at two different concentrations of thrombin, a low and a high, were used.

The fibrin glue having the low thrombin concentration was used as a first layer of the double coating.

The fibrin glue having the high thrombin concentration, applied as "second layer," plays the role of mechanical barrier which entirely covers the injury and the first layer. The kinetic of fibrin formation of the second layer is faster than that of the first one and also the physical properties of the two layers are different.

1.10 Mode of Application

Caecum was abraded and the parietal wall was incised, respectively, and covered with a single or a double coating of fibrin glue (FG) depending on the protocol design.

Fibrin glue can be applied sequentially or simultaneously on both injured surfaces.

By combining the application type (single or double) and the application mode (sequential or simultaneous), four different cases can be obtained (cf. FIG. 5).

Case 1 single coating & sequential application

Case 2 double coating & sequential application

Case 3 single coating & simultaneous application

Case 4 double coating & simultaneous application

(Case 2 will not be tested herein. Case 3 represents the "in vivo" conditions for the adhesion development.)

2. EXAMPLES

Example 1

Preparation of a Fibrin Film Using a Dual Syringe Device

The fibrin films were casted by using a commercial dual syringe device, not of the present invention, after reconstitution of the vials of a commercial fibrin glue kit in accordance with the information in the instrucion leaflet of the respective kit employed. By way of example, a two-component fibrin glue kit comprising the following constituents may be used:

Vial (1)	Human topical fibrinogen complex (dry concentrate)	
	protein	10–13 g/100 ml
	clottable protein	80% minimum
	albumin (human)	0.5–1.5 g/100 ml
	plasminogen	0.05 mg/ml maximum
	Factor XIII	10–40 IU/ml
	polysorbate-80	0.3% (w/v) maximum
	pH	7.1–7.5
Vial (2)	Sterile water (3.5 ml) for reconstituting the content of vial (1) at 37° C. in a water bath	
Vial (3)	Human thrombin	
	potency	300 ÷ 50 IU/ml
	albumin (human)	0.05 ÷ 0.01 g/ml
	glycine	0.30M ÷ 0.05M
	pH	6.5–7.1
Vial (4)	35–45 mM $CaCl_2$ (3.5 ml) for reconstituting the content of vial (3) at room temperature	

After reconstitution, the fibrinogen-containing solution was kept at room temperature. Further thrombin dilutions were made with 20 mM $CaCl_2$ as diluent. Using the dual syringe device, not of the present invention, the mixture "Fibrinogen-Thrombin" was applied to a petri dish, while care was taken that at any time equal amounts of the fibrinogen-containing solution and the thrombin-containing solution were pressed out of the respective syringe. With low concentrations of thrombin, the petri dish was tilted to cover the surface with a fibrin glue of regular and homogenous thickness. With high concentrations of thrombin, particular care was taken that from the beginning the mixture of the fibrinogen-containing solution and the thrombin-containing solution was uniformly spread over the surface of the petri dish. The petri dish was incubated at 37° C. for two hours.

Optionally, disinfectants, e.g. methylene blue in a concentration of 10 mg/l–10 g/l or drugs, may be dissolved in the contents of vials (2) or (4) before these are used for reconstituting the contents of vials (1) and (2), respectively.

The Fibrin film obtained may be air-dried and rehydrated before use. If the thrombin solution did not already comprise substances, like disinfectants or drugs for enhancing the desired therapeutic effect on the fibrin film, the solution used for rehydration may include those substances.

Example 2

Preparation of a Fibrin Film Using a Dry Fibrinogen Sheet

The solutions given in Example 1 were used with the following modifications.

3.5 ml of the reconstituted fibrinogen-containing solution were poured in a petri dish of 51 mm diameter which was tilted to spread the material all over the entire surface. The water contained in the fibrinogen-containing solution was evaporated by air drying. Thus, a dry fibrinogen sheet having a thickness of 100 μm and a weight of 0.4291 g was obtained. 3.5 ml of a reconstituted thrombin-containing solution were poured into the petri dish containing the dry, sheet-like fibrinogen material. The reaction mixture was then kept at 37° C. for 2 hours. The fibrin film thus obtained may either directly be used or be dried and rehydrated before use. Alternatively, the dry, sheet-like fibrinogen material may be converted into a fibrin film only before use.

Both the dry, sheet-like fibrinogen material and the dried fibrin film may be included in a commercial kit further comprising ancillary components for processing and rehydration, respectively, of the sheet-like materials.

Example 3

Preparation of a Fibrin Sealant/Glue

The preparation of a fibrin glue was performed as per the technical information of the instruction leaflet provided with the kit employed. The fibrin glue was prepared extemporaneously at different concentrations of thrombin, e.g. 4, 5, 20, 100, 150, and 300 IU/ml and used as described hereinbelow.

Example 4

"Control" Group

The animals were operated to develop adhesion, and therefore no treatment had been carried out. Accordingly, hemostasis was not achieved, and, thus, all conditions to develop with an incidence of 100% severe type 3 adhesions between the caecum and the parietal wall were present.

The results are reported in the following Table 1:

TABLE 1

Control Group

No.*	Products Applied	Adhesion: Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet
1	—/—/—	(3)	(Ui) (1)	—	—
2	—/—/—	(3)	—	(Ui) (1)	(Om) (1)
3	—/—/—	(3) (xx)	—	—	—
4	—/—/—	(3)	—	(Ui) (1)	(Ui) (1)
5	—/—/—	(3) (xx)	—	—	—
6	—/—/—	(3) (xx)	—	—	—
7	—/—/—	(3)	—	—	—
8	—/—/—	(3) (xx)	—	—	—
9	—/—/—	(3)	—	—	—
10	—/—/—	(3)	—	—	(Bl) (1)
11	—/—/—	(3)	—	—	—
12	—/—/—	(3)	—	—	(Om) (1)
13	—/—/—	(3)	—	—	—
14	—/—/—	(3)	—	(Om) (1)	(Om, Ui) (1, 1)
15	—/—/—	(3)	—	—	—
16	—/—/—	(3)	—	—	—
17	—/—/—	(3)	—	—	—
18	—/—/—	(3)	—	—	—
19	—/—/—	(3)	—	—	—
20	—/—/—	(3)	—	—	—
21	—/—/—	(3)	—	—	—

* = Number of animal (female Wistar rat)
(x) = a piece of fibrin was still present

Example 5

Fibrin Film Application

As a rule the fibrin films used were made in accordance with Example 1.

Example 5a

Sham Control with Fibrin Film (FF)

Two animals were used as sham control. One received a fibrin film made using 3 IU thrombin and with water as diluent (FF 3 IU), the other received fibrin film made using 20 IU thrombin and with 20 mM $CaCl_2$ as diluent (FF 20 IU). No injuries were induced on the caecum and peritoneum.

No adhesions were observed with both animals. The results are reported in the following Table 2:

TABLE 2

Sham Control with Fibrin Film

Product No.*	Applied	Adhesion: Pat. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.	Fat & Visc.	Fat & Fat
1	SHAM (FF 20 IU)	—	—	—	—	—	—
2	SHAM (FF 3 IU)	—	—	—	—	—	—

*= Number or animal (female Wistar rat)

Example 5b

Application of a Fibrin Film without Control of Hemostasis

Fibrin films FF 3 IU and FF 20 IU in accordance with Example 5a were used as mechanical barrier without controlling hemostasis on the caecum and parietal injuries. Alternatively, a Fibrin Film 300 IU was used as mechanical barrier.

The animal sacrificed after 10 days showed medium caeco-parietal adhesions (type 2) and large number of fat adhesions, involving mainly uterus and bladder. Surprisingly and most importantly, the animals treated with FF 300 IU virtually did not develop any adhesions.

The results are reported in the following Table 3:

TABLE 3

No.*	Product Applied	Fibrin Film Alone — Adhesion: Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.	Fat & Visc.	Fat & Fat
1	SHAM (FF 20 IU)	—°	—°	—°	—°	—°	—°
2	SHAM (FF 3 IU)	—°	—°	—°	—°	—°	—°
3	FF 3 IU	—	—	(Ut) (2)	(Ut, Bl) (3,2)	—	—
4	FF 3 IU	(2)	—	(Om, Ut) Bl) (2, 2,2)	(Om) (2)	—	—
5	FF 3	(2)	—	(Ut) (2)	—	—	—
6	FF 3	(1)	—	(Ut, Bl) (2,2)	(Ut) (2)	—	—
7	FF 20 IU	(2)	—	(Ut) (2)	(Ut(2)	—	—
8	FF 20 IU	(3)	—	—	—	—	—
9	FF 20 IU	(3)	—	(Ut) (1)	—	—	—
10	FF 20 IU	(1)	—	—	—	—	—
11	FF 300 IU	—	—	—	—	—	—
12	FF 300 IU	—	—	(Ut) (1)	—	—	—
13	FF 300 IU	—	—	—	—	—	—
14	FF 300 IU	—	—	—	—	—	—

*= Number of animal (female Wistar rat)
°= Necropsy after 3 days/others after 10 days

The fibrin film in accordance with the invention which was used for the treatment of animal no. 11 was obtained by an alternative process as follows:

The first, fibrinogen-containing solution was poured in a petri dish having a diameter of 91 mm. The temperature of said solution was decreased by incubating the petri dish for a few minutes at low temperature, here for 4 min. at −12° C. Then the second, thrombin-containing solution (RT) was added and mixed with the first solution. The petri dish was incubated until completion of the conversion of fibrinogen to fibrin, here for 24 hours at 37° C.

Example 6

Fibrin Glue "Single Coating"

Using thrombin-containing solutions comprising 4 IU/ml (cf. Example 6a) and 100 IU/ml thrombin (cf. Example 6b), fibrin glues were applied as hemostatic agent to the abraded caecum and the incised peritoneum each. By way of example, this is shown in the following Table as follows: FG 4 IU / - - - - / FG 4 IU; where (- - - -) indicates that no fibrin film is placed between the injuries. The fibrin glue was applied to the injuries in sequence. The waiting time to allow a setting of the fibrin glue was 5 minutes after each application.

In Example 6a, severe type 3 adhesions between the caecum and the parietal wall were observed.

The results are shown in the following Table 4:

TABLE 4

Single Coating, Sequential Application Time: 5 min

No.*	Products Applied	Adhesion: Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 4 IU / - - - -/ FG 4 IU / - - - -/	(3)	—	—	—
2	FG 4 IU / - - - -/ FG 4 IU / - - - -/	(2)	—	—	—
3	FG 4 IU / - - - -/ FG 4 IU / - - - -/	(3)	—	—	—
4	FG 4 IU / - - - -/ FG 4 IU / - - - -/	(3)	—	—	—
5	FG 4 IU / - - - -/ FG 4 IU / - - - -/	(3)	—	—	—

*= Number of animal (female Wistar rat)

In Example 6b, two animals developed severe type 3 adhesions between caecum and paricetal wall. The caecum was partly included into the parietal wall. Two animals did not develop adhesion.

The results are reported in the following Table 5:

TABLE 5

Single Coating, Sequential Application Time: 5 min

No.*	Products Applied	Adhesion: Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 100 IU / - - - -/ FG 100 IU / - - - -/	(3)	—	—	(Om, Ut) (1, 1)
2	FG 100 IU / - - - -/ FG 100 IU / - - - -/	—	—	—	—
3	FG 100 IU / - - - -/ FG 100 IU / - - - -/	—	—	—	—
4	FG 100 IU / - - - -/ FG 100 IU / - - - -/	(3)	—	—	—

*= Number of animal (female Wistar rat)

Example 7

Fibrin Glue "Double Coatings"

Fibrin glues were applied at two different thrombin concentrations.

Example 7a

A first layer of a fibrin glue made using a thrombin-containing solution comprising 4 IU/ml thrombin (-G 4 IU) was simultaneously (i.e., only with a minimal delay caused by the handling) applied to both the abraded caecum and the incised parietal wall.

After a waiting time of 5 minutes to allow the polymerization, a second layer of fibrin glue 100 IU, FG 100 IU, was simultaneously applied on the same organs. This was followed by a second waiting time of 5 minutes before the surgical procedure was continued.

One animal did not develop adhesion. With one animal, which developed a severe type 3 adhesion, a remaining piece of fibrin was observed. Three animals developed mild type 1 adhesions between the caecum and the parietal wall.

The results are reported in the following Table 6:

TABLE 6

Double coating with FG 4 IU and FG 100 UI,
Simultaneous Application Time: 5 Minutes

No.*	Products Applied	Adhesion Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 4 & 100 IU / - - - -/ FG 4 & 100 IU / - - - -/	(3) (x)	—	—	(Om, Ut) (1,1)
2	FG 4 & 100 IU / - - - -/ FG 4 & 100 IU / - - - -/	(1)	—	—	—
3	FG 4 & 100 IU / - - - -/ FG 4 & 100 IU / - - - -/	(1)	—	—	—
4	FG 4 & 100 IU / - - - -/ FG 4 & 100 IU / - - - -/	—	—	—	—
5	FG 4 & 100 IU / - - - -/ FG 4 & 100 IU / - - - -/	(1)	—	—	—

*= Number of animal (female Wistar rat)
x= a piece of fibrin was still present

Example 7b

A parallel experiment under the same conditions was conducted, but with the single exception that higher thrombin concentrations (FG 5 IU and FG 150 IU) were used.

Four animals did not develop adhesion. With one animal, which developed a mild type 2 adhesion between the caecum and the parietal wall, a remaining piece of fibrin was observed.

The results are reported in the following Table 7:

TABLE 7

Double coating with FG 5 IU and FG 150 UI,
Simultaneous Application Time: 5 Minutes

No.*	Products Applied	Adhesion Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 5 & 150 IU / - - - -/ FG 5 & 150 IU / - - - -/	(2) (x)	—	—	—
2	FG 5 & 150 IU / - - - -/ FG 5 & 150 IU / - - - -/	—	—	—	—
3	FG 5 & 150 IU / - - - -/ FG 5 & 150 IU / - - - -/	—	—	—	—
4	FG 5 & 150 IU / - - - -/ FG 5 & 150 IU / - - - -/	—	—	—	—

*= Number of animal (female Wistar rat)
x= a piece of fibrin was still present

Example 8

Sandwich Method—Use of a Combination of a Fibrin Glue and a Fibrin Film

The sandwich method combines the use of a fibrin glue as hemostatic agent/wound repair promoter and of a fibrin film as mechanical barrier. Three types of fibrin film which had been made using 4 IU, 20 IU and 300 IU thrombin in accordance with Example 1 were used. Due to the different thrombin concentrations of the respective fibrin films, the time required for complete fibrinogen-fibrin conversion varied. However, this is of no importance as the films were kept at 37° C. for more than two hours, a time greater than that required as determined theoretically and practically by means of turbidity measurement.

Example 8a

A fibrin glue 100 IU used as hemostatic agent was applied simultaneously both to the abraded caecum and the incised parietal wall with a waiting time of 5 minutes. In parallel (cf. (ii)), it was applied sequentially to the abraded caecum and the incised parietal wall with a waiting a time of 7 minutes each time. In both experiments, a fibrin film of 4 IU was used.

(i) Simultaneous application time: 5 minutes

Two animals did not develop adhesions between caecum and parietal wall, while two animals (developed mild type 1 adhesions between these surfaces. One animal developed a type 2 caeco-parietal adhesion.

The results are reported in the following Table 8:

TABLE 8

Sandwich Method, Simultaneous Application Time: 5 Minutes

No.*	Products Applied	Adhesion Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 100 IU / - - - -/ FG 100 IU / - - - -/	—	(Ut(1)	—	—
2	FG 100 IU / - - - -/ FG 100 IU / - - - -/	(1)	—	—	—
3	FG 100 IU / - - - -/ FG 100 IU / - - - -/	(1)	—	—	—
4	FG 100 IU / - - - -/ FG 100 IU / - - - -/	—	—	—	—
5	FG 100 IU / - - - -/ FG 100 IU / - - - -/	(1)	—	—	—

*= Number of animal (female Wistar rat)

(ii) Sequential application time: 7 minutes

Virtually none of the animals developed adhesion. The results are reported in the following Table 9:

TABLE 9

Sandwich Method, Sequential Application Time: 7 Minutes

No.*	Products Applied	Adhesion Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 100 IU/FF 4 IU/ FG 100 IU	—	—	—	—
2	FG 100 IU/FF 4 IU/ FG 100 IU	—	—	—	—
3	FG 100 IU/FF 4 IU/ FG 100 IU	—	—	—	(Bl) (1)
4	FG 100 IU/FF 4 IU/ FG 100 IU	—	—	—	—
5	FG 100 IU/FF 4 IU/ FG 100 IU	—	—	—	—

*= Number of animal (female Wistar rat)

Example 8b

In another experiment, a fibrin glue 100 IU and a fibrin film 20 IU were used in combination. The fibrin glue was sequentially applied to the abraded caecum and to the incised parietal wall with a waiting time of 7 minutes each.

None of the animals developed adhesion.

The results are reported in the following Table 10:

TABLE 10

Sandwich Method, Sequential Application Time: 7 Minutes

No.*	Products Applied	Adhesion Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 100 IU/FF 20 IU/ FG 100 IU	—	—	—	—
2	FG 100 IU/FF 20 IU/ FG 100 IU	—	—	—	—
3	FG 100 IU/FF 20 IU/ FG 100 IU	—	—	—	—

*= Number of animal (female Wistar rat)

Example 8c

In a further experiment, a fibrin gluc 100 IU was combined with a fibrin film 300 IU. The fibrin film of the invention was obtained in accordance with the alternative method mentioned in Example 5 (cf. animal no. 11), but with an incubation of 10 min. at −12° C. The fibrin film thus obtained was air dried and then rehydrated before being used like a fibrin film made in accordance with Examples 1 and 2. As in Example 8b, the fibrin glue was sequentially applied to the abraded caecum and the incised parietal wall with an application time of 7 minutes each.

One animal developed a very mild adhesion. The other four animals did not develop any adhesions.

The results are summarized in the following Table 11:

TABLE 11

Sandwich Method, Sequential Application Time: 7 Minutes

No.*	Products Applied	Adhesion Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 100 IU/FF 300 IU(*)/ FG 100 IU	(<1)	—	—	—
2	FG 100 IU/FF 300 IU(*)/ FG 100 IU	—	—	—	—
3	FG 100 IU/FF 300 IU(*)/ FG 100 IU	—	—	—	—
4	FG 100 IU/FF 300 IU(*)/ FG 100 IU	—	—	—	—
5	FG 100 IU/FF 300 IU(*)/ FG 100 IU	—	—	—	—

*= Number of animal (female Wistar rat)

Example 8d

In addition, experiments were performed using a fibrin film FG 20 IU in combination with fibrin films of 20 IU and 300 IU, respectively. The fibrin films were made in accordance with Example 1, air-dried and then rehydrated before use. As in Example 8c, the fibrin glue was sequentially applied to the abraded caecum and the incised parietal wall with a waiting time/application time of 7 minutes each.

None of the animals developed any adhesions.

The results are summarized in the following Table 12:

TABLE 12

Sandwich Method, Sequential Application Time: 7 Minutes

No.*	Products Applied	Adhesion Par. & Caec.	Visc. & Caec.	Fat & Caec.	Fat & Pariet.
1	FG 20 IU/FF 20 IU/FG 20 IU	—	—	—	—
2	FG 20 IU/FF 20 IU/FG 20 IU	—	—	—	—
3	FG 20 IU/FF 20 IU/FG 20 IU	—	—	—	—
4	FG 20 IU/FF 20 IU/FG 20 IU	—	—	—	—

*= Number of animal (female Wistar rat)

3. Discussion

3.1 Application of a Fibrin Film without Control of Hemostasis

As shown in Example 3b, fibrin films made by using 3 IU and 20 IU, if used in an uncontrolled hemostatic environment, did not prevent adhesion formation. On the other hand, the use of fibrin films with high thrombin concentration completely prevented adhesion formation without hemostasis being performed.

3.2 Single Coating

Sequential application of FG 4 IU (cf. Example 6a) did not prevent adhesion formation at all, whereas sequential application of FG 100 IU (cf. Example 6b) allowed a 50% prevention of adhesion formation. These findings suggest that the conversion of fibrinogen to fibrin was not complete on both caecum and parietal wall.

3.3 Double Coating

The simultaneous application of FG 4 & 100 for five minutes was still too short to prevent adhesion formation, but decreased the adhesion grade and tensile properties (cf. Example 7a).

In Example 7b, rather than to increase the simultaneous application time/waiting time for achieving complete fibrinogen-fibrin conversion, the thrombin concentrations were increased in order to reduce the clotting time. In fact, with the simultaneous application of FG 5 & 150 for five minutes, the number of adhesions was decreased.

The presence of a remaining fibrin piece in Examples 7a and 7b indicates, however, that the application volume had to be better controlled. It also has to be pointed out that the double coating of fibrin glues was applied to an injured area where the fibrinolytic system was dramatically impaired. A more controlled delivery (by better handling) and a lower volume of fibrin glue at a higher thrombin concentration (to achieve a faster and more complete conversion of fibrinogen to fibrin) seem to be more suitable for improving the outcome.

3.4 Sandwich Method Using a Fibrin Glue and a Fibrin Film

As shown in Table 8, simultaneous application of FG 100 IU/FF 4 IU/FG 100 IU for five minutes did not totally prevent adhesion formation. The fibrin film made by using 4 IU thrombin is induced a stabilized film having a complete fibrinogen-fibrin conversion, but the inventors are aware that such a fibrin film has particularly large and opened pores. On the other hand, Table 8 shows that FG 100 IU, applied with a waiting time of five minutes, did not completely prevent the development of adhesions. Thus, it may be that FG 100 IU had not reached completion of the conversion of fibrinogen to fibrin and interacted with FF 4 IU in such a manner that no complete prevention of adhesion formation was achieved.

Principally, this could be avoided by either increasing the thrombin concentration of the fibrin glue used (to achieve a

faster clotting) or by increasing its sequential application time (to provide more time for the fibrinogen-fibrin conversion), or by increasing the thrombin concentration of the fibrin film (to produce smaller pores).

As shown in Table 9, when increasing the sequential application time up to seven minutes, the application of FG 100 IU/FF 4 IU/FG 100 IU prevented adhesion formation. Likewise, adhesion formation was prevented by increasing the thrombin concentration of the fibrin film to 20 IU as can be taken from Table 10.

It appeared interesting to test a fibrin film made by using a very high thrombin concentration (300 IU). In this case (cf. Table 11), the first coating to control hemostasis (FG 100 IU) as well as application mode and time were maintained in order to compare this situation with the previous examples. In the light of the present disclosure (cf. Table 12), those of skill in the art will appreciate that different combinations may lead to excellent results in the prevention of adhesion formation. Finally, it is pointed out that interestingly the fibrin film FF 300 IU completely prevented adhesion formation without hemostasis having been performed, while this was not achieved by using FF 20 IU. The above-described experiments indicate that with the sandwich technique even fibrin films having a pore size above 5 μm may be used, while the fibrin films preferably should have a pore size below 5 μm when used alone, i.e., without control of hemostasis.

Example 9

Using the Fibrin Delivery Device of the Present Invention

Fibrin Film 1: A fibrin film was formed by simultaneously spraying onto a plastic petri dish 1 ml of a liquid solution of fibrinogen having a concentration of 50 mg/ml and 1 ml of a thrombin concentration of 100 IU/ml. The Fibrin Film upon visual inspection appeared to have a regular, homogenous structure.

Fibrin Film 2: A fibrin film was formed by sequential spraying onto a plastic petri dish a fibrinogen solution having a concentration of 50 mg/ml and a thrombin concentration of 100 IU/ml. The sequential spraying including spraying 1 ml of thrombin followed by 1 ml of fibrinogen. The Fibrin Film upon visual inspection appeared to have a regular, homogenous structure.

While specific embodiments have been illustrated and described, numerous modifications are possible without departing from the spirit of the invention, and the scope of protection is only limited by the scope of the accompanying claims.

We claim:

1. A medical device for laparoscopically delivering volumetric quantities of a first and a second biochemically reactive fluid comprising:

a first container having a first fluid opening, the first container being adapted to contain the first biochemically reactive fluid;

a second container having a second fluid opening adjacent the first fluid opening, the second container being adapted to contain the second biochemically reactive fluid;

means for separately atomizing the first and second biochemically reactive fluids into an aerosol with at least one energy source selected from the group consisting of a liquid energy, a mechanical energy, a vibration energy, and an electric energy;

a fluid pressurizer for pressurizing the first and the second biochemically reactive fluids for delivery under pressure through a spray unit onto a surface; and

wherein the first and second biochemically reactive fluids first mix on the surface.

2. The device of claim **1** wherein the first and second containers are each a syringe.

3. The device of claim **2** wherein the first syringe and the second syringe are substantially of the same volume.

4. The device of claim **1** wherein the first and second containers are pipettes.

5. The device of claim **4** wherein each of the first and second fluid pressurizer is a plunger.

6. The device of claim **1** wherein the means for separately atomizing the first and second biochemically reactive fluids into an aerosol is a spray unit capable of delivering the first and second biochemically reactive fluids without clogging.

7. A medical device for laparoscopically delivering volumetric quantities of a first and a second biochemically reactive fluid comprising:

a first container having a first fluid opening, the first container being adapted to contain the first biochemically reactive fluid;

a second container having a second fluid opening adjacent the first fluid opening, the second container being adapted to contain the second biochemically reactive fluid;

a spray unit in fluid communication with the first container and the second container, the spray unit being capable of separately atomizing the first and second biochemically reactive fluids into an aerosol with an energy selected from the group consisting of liquid energy, mechanical energy, vibration energy, and electric energy;

a fluid pressurizer for pressurizing the first and the second biochemically reactive fluids for delivery under pressure through the spray unit onto a surface; and

wherein the first and second biochemically reactive fluids first mix on the surface.

* * * * *

THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Appellant(s):	Smith et al.
Appl. No.:	10/634,663
Conf. No.:	6356
Filed:	August 5, 2003
Title:	IN VITRO CELL CULTURE EMPLOYING A FIBRIN NETWORK IN A FLEXIBLE GAS PERMEABLE CONTAINER
Art Unit:	1797
Examiner:	Nathan Andrew Bowers
Docket No.:	WM-5934 (R1) US (112713-983)

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANTS' APPEAL BRIEF

Sir:

Appellants submit this Appeal Brief in support of the Notice of Appeal filed on August 15, 2008. This Appeal is taken from the Final Rejection in the Office Action dated June 5, 2008.

I. REAL PARTY IN INTEREST

The real party in interest for the above-identified patent application on Appeal is Baxter International Inc., by virtue of an Assignment recorded on December 18, 2003 at reel 014207, frames 0406-0408 in the United States Patent and Trademark Office.

II. RELATED APPEALS AND INTERFERENCES

Appellants' legal representative and the Assignees of this patent application do not know of any prior or pending appeals, interferences or judicial proceedings that may be related to, directly affect or be directly affected by or have a bearing on the Board's decision with respect to the above-identified Appeal.

III. STATUS OF CLAIMS

Claims 1, 3-11, 19-21 and 23-53 are pending in this application. Claims 2, 12-18, 22 and 54-120 were previously canceled. Claims 1, 3-11, 19-21 and 23-53 stand rejected. Therefore, Claims 1, 3-11, 19-21 and 23-53 are being appealed in this Brief. A copy of the appealed claims is included in the Claims Appendix.

IV. STATUS OF AMENDMENTS

A non-final Office Action was mailed on November 26, 2007 rejecting the claims as obvious in view of several cited references. Appellants responded to the non-final Office Action on February 26, 2008 without amending the claims to overcome the obvious rejections set forth in the non-final Office Action. A final Office Action maintaining the rejections was mailed on June 5, 2008. Appellants filed a Notice of Appeal on August 15, 2008. A copy of the non-final Office Action and final Office Action are attached as Exhibits A and B, respectively, in the Evidence Appendix.

V. SUMMARY OF CLAIMED SUBJECT MATTER

A summary of the claimed subject matter by way of reference to the specification and/or figures for each of the independent claims is provided as follows:

Independent Claim 1 is directed a cell culture container (page 1, lines 7-11; page 4, lines 16-18; page 6, lines 28-31; Figure 1b) comprising a closed supporting container (page 5, lines 9-12; page 6, lines 28-31) comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area (page 4, lines 19-26; page 9, line 26 to page 10, line 30), the first side wall being constructed from a gas permeable material selected from the group consisting of polymeric material, paper, and fabric (page 4, lines 19-26; page 4, line 30 to page 5, line 1; page 7, line 10 to page 8, line 21), the first side wall having a gas permeability sufficient to permit cellular respiration (page 4, lines 19-27; page 8, lines 16-21), and the second side wall being constructed from a material selected from the group consisting of polymeric material, paper, fabric, and foil (page 4, lines 19-26; page 4, line 30 to page 5, line 1; page 7, line 10 to page 8, line 21), wherein at least one of the first and the second side wall comprises an interior surface comprising an ethylene vinyl acetate copolymer (page 5, line 1; page 7, lines 14-19; page 8, line 22 to page 9, line 5); and a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container (page 4, lines 27-29; page 5, lines 5-8; page 13, line 2 to page 14, line 31).

Independent Claim 48 is directed to a cell culture container (page 1, lines 7-11; page 4, lines 16-18; page 6, lines 28-31; Figure 1b) comprising a closed supporting container (page 5, lines 9-12; page 6, lines 28-31) comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area (page 4, lines 19-26; page 9, line 26 to page 10, line 30), each side wall having an interior surface, the first side wall and the second side wall comprising an interior surface constructed from an ethylene vinyl acetate copolymer having a gas permeability sufficient to permit cellular respiration (page 5, line 1; page 7, lines 14-19; page 8, line 22 to page 9, line 5); and a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container (page 4, lines 27-29; page 5, lines 5-8; page 13, line 2 to page 14, line 31).

Independent Claim 51 is directed to a cell culture container (page 1, lines 7-11; page 4, lines 16-18; page 6, lines 28-31; Figure 1b) comprising a closed supporting container (page 5, lines 9-12; page 6, lines 28-31) comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area (page 4, lines 19-26; page 9, line 26 to page 10, line 30), the side walls are constructed from a multilayer gas permeable polymeric structure having a gas permeability sufficient to permit cellular respiration (page 4, lines 19-27; page 8, lines 16-21), and the multilayer polymeric structure comprising: a first layer comprising polystyrene having a thickness within the range of 0.0001 inches to about 0.0010 inches (page 9, lines 6-11); and a second layer adhering to the first layer (page 4, lines 19-26; page 9, line 26 to page 10, line 30), the second layer comprising an interior surface comprised of an ethylene vinyl acetate copolymer (page 5, line 1; page 7, lines 14-19; page 8, line 22 to page 9, line 5), the second layer having a thickness within the range of 0.004 inches to about 0.025 inches (page 9, lines 11-19); and a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container (page 4, lines 27-29; page 5, lines 5-8; page 13, line 2 to page 14, line 31).

Although specification citations are given in accordance with 37 C.F.R. §1.192(c), these reference numerals and citations are merely examples of support in the specification for the terms used in this section of the Brief. There is no intention to suggest in any way that the terms of the claims are limited to the examples in the specification. As demonstrated by the references numerals and citations, the claims are fully supported by the specification as required by law. However, it is improper under the law to read limitations from the specification into the claims. Pointing out specification support for the claim terminology in accordance with Rule 1.192(c) does not in any way limit the scope of the claims to those examples from which they find support. Nor does this exercise provide a mechanism for circumventing the law precluding reading limitations into the claims from the specification. In short, the references numerals and specification citations are not to be construed as claim limitations or in any way used to limit the scope of the claims.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Claims 1, 3-11, 19-21, 23-34 and 48-53 stand rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 5,935,847 to Smith et al. ("*Smith*") in view of either U.S. Patent No. 6,759,245 to Toner et al. ("*Toner*") or U.S. Patent No. 5,912,177 to Turner ("*Turner*") and further in view of U.S. Patent No. 5,686,304 to Codner ("*Codner*"). Copies of *Smith*, *Toner*, *Turner* and *Codner* are attached hereto as Exhibits C, D, E and F, respectively, in the Evidence Appendix.
2. Claims 36-47 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over *Smith* in view of *Toner*/*Turner* and *Codner* in further view of U.S. Patent No. 5,989,215 to Delmotte ("*Delmotte*"). A copy of *Delmotte* is attached hereto as Exhibit G.

VII. ARGUMENT

A. LEGAL STANDARDS

Obviousness under 35 U.S.C. §103

The Federal Circuit has held that the legal basis for a determination of obviousness under 35 U.S.C. § 103 is:

> whether the claimed invention as a whole would have been obvious to a person of ordinary skill in the art at the time the invention was made...The foundational facts for the prima facie case of obviousness are: (1) the scope and content of the prior art; (2) the difference between the prior art and the claimed invention; and (3) the level of ordinary skill in the art...Moreover, objective indicia such as commercial success and long felt need are relevant to the determination of obviousness...Thus, each obviousness determination rests on its own facts.

In re Mayne, 41 U.S.P.Q. 2d 1451, 1453 (Fed. Cir. 1997).

In making this determination, the Examiner has the initial burden of proving a *prima facie* case of obviousness. *In re Rijckaert*, 9 F.3d 1531, 1532, 28 U.S.P.Q. 2d 1955, 1956 (Fed. Cir. 1993). This burden may only be overcome "by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings." *In re Fine*, 837 F.2d 1071, 1074, 5 U.S.P.Q. 2d 1596, 1598 (Fed. Cir. 1988). "If the examination at the initial stage does not produce a prima facie case of unpatentability, then without more the applicant is entitled to grant of the patent." *In re Oetiker*, 24 U.S.P.Q. 2d 1443, 1444 (Fed. Cir. 1992).

Moreover, the Examiner must provide explicit reasons why the claimed invention is obvious in view of the prior art. The Supreme Court has emphasized that when formulating a rejection under 35 U.S.C. § 103(a) based upon a combination of prior art elements it remains necessary to identify the reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed. *KSR v. Teleflex*, 127 S. Ct. 1727 (2007).

Of course, references must be considered as a whole and those portions teaching against or away from the claimed invention must be considered. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve Inc.*, 796 F.2d 443 (Fed. Cir. 1986). "A prior art reference may be considered to teach away when a person of ordinary skill, upon reading the reference would be discouraged

from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the Applicant." *Monarch Knitting Mach. Corp. v. Fukuhara Indus. Trading Co., Ltd.*, 139 F.3d 1009 (Fed. Cir. 1998) (quoting *In re Gurley*, 27 F.3d 551 (Fed. Cir. 1994)).

B. THE CLAIMED INVENTION

There are three independent claims on appeal: Claims 1 and 48 and 51. Independent Claim 1 is generally directed a cell culture container comprising a closed supporting container comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area. The first side wall is constructed from a gas permeable material selected from the group consisting of polymeric material, paper, and fabric. The first side wall has a gas permeability sufficient to permit cellular respiration. The second side wall is constructed from a material selected from the group consisting of polymeric material, paper, fabric, and foil. At least one of the first and the second side wall comprises an interior surface comprising an ethylene vinyl acetate copolymer. The cell culture container further comprises a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container.

Independent Claim 48 is generally directed to a cell culture container comprising a closed supporting container comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area. Each side wall has an interior surface. The first side wall and the second side wall comprise an interior surface constructed from an ethylene vinyl acetate copolymer having a gas permeability sufficient to permit cellular respiration. The cell culture container further comprises a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container.

Independent Claim 51 is generally directed to a cell culture container comprising a closed supporting container comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area. The side walls are constructed from a multilayer gas permeable polymeric structure having a gas permeability sufficient to permit cellular respiration. The multilayer polymeric structure

comprises a first layer comprising polystyrene having a thickness within the range of 0.0001 inches to about 0.0010 inches and a second layer adhering to the first layer. The second layer comprises an interior surface comprised of an ethylene vinyl acetate copolymer. The second layer has a thickness within the range of 0.004 inches to about 0.025 inches. The cell culture container further comprises a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container.

C. THE REJECTION OF CLAIMS 1, 3-11, 19-21, 23-34 AND 48-53 UNDER 35 U.S.C. §103(A) TO *SMITH*, *TONER*, *TURNER* AND *CODNER* SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A *PRIMA FACIE* CASE OF OBVIOUSNESS WITH RESPECT TO CLAIMS 1, 3-11, 19-21, 23-34 AND 48-53

1. *Smith*, *Toner*, *Turner* and *Codner* alone or in combination fail to disclose or suggest every element of the claimed invention

Independent Claims 1, 48 and 51 recite, in part, a closed supporting container comprising a side wall having a flexible interior surface comprising an ethylene vinyl acetate copolymer. A fibrin matrix layer is on a portion of the interior surface of the side wall of the supporting container. In contrast, even if combined, *Smith*, *Toner*, *Turner* and *Codner* fail to disclose or suggest every element of independent Claims 1, 48 and 51.

The present claims provide an in vitro cell culture employing a fibrin network in a flexible container. Appellants have surprisingly found that providing the flexible container with an interior surface of a portion of the side walls constructed from an ethylene vinyl acetate copolymer having a fibrin matrix presents an environment conducive to adherent cell proliferation and maturation. By incorporating a fibrin matrix in a flexible cell culture container, the fibrin matrix lessens the functional biocompatibility requirements of the materials from which the container is fabricated.

By transferring the biocompatibility requirement of the culture from the container to the fibrin matrix, the material selection of the container can focus on other attributes, such as gas permeability, optical clarity, and material strength. Accordingly, the flexible container having an

interior surface comprising an ethylene vinyl acetate copolymer is well suited for applications involving therapeutic transplantation of cultured cells. For example, the container is permeable to gases, but not vented, thereby maintaining an environment free of contaminants during cell culture and processing. This allows for an improved flexible, gas permeable container in accordance with embodiments of the present claims that is suitable for culturing anchorage dependent mammalian cells for expansion and transplantation, which had previously been done only using rigid, gas impermeable cell culture flasks or plates.

Even if combined, *Smith*, *Toner*, *Turner* and *Codner* fail to disclose or suggest a number of elements of independent Claims 1, 48 and 51. *Smith*, *Toner* and *Turner* fail to disclose or suggest a flexible interior surface comprising an ethylene vinyl acetate copolymer as required by Claims 1, 48 and 51. *Smith*, *Toner*, *Turner* and *Codner* also fail to disclose or suggest a fibrin matrix layer on a portion of the ethylene vinyl acetate copolymer interior surface as required by Claims 1, 48 and 51.

In an attempt to arrive at the present claims, the Examiner alleges that *Smith's* container comprises an ethylene vinyl acetate copolymer as a sidewall. See final Office Action, page 3, paragraph 2. Nevertheless, Appellants respectfully submit that *Smith* only teaches using ethylene vinyl acetate as a substrate layer 14, which represents the outer layer of *Smith's* container. See *Smith*, column 5, lines 40-45, and Figures 1-6 and 11a-b. *Smith* teaches that the first layer 12 actually represents the interior layer of the container.

Appellants also respectfully submit that *Smith* is entirely directed to an interior cell growth layer composed of polystyrene and exterior layer composed of a polymeric layer comprising a multiple component polymer alloy blend. For example, *Smith* teaches that the first layer 12 of the films forms an inner cell growth surface. See *Smith*, column 3, line 59 to column 4, line 10. *Smith* teaches that the first layer 12 is an ultra-thin layer of polystyrene. *Id.* *Smith* never discloses using ethylene vinyl acetate copolymer as any part of the inner cell growth surface layer anywhere in his disclosure.

Toner is directed to flow-through modular cell culture devices including one or more flat-plate modules. See *Toner*, column 2, lines 35-45. *Toner* is relied upon only for the teaching that a fibrin matrix may be used to accommodate cell growth. Nevertheless, *Toner* fails to disclose or suggest a flexible interior surface comprising an ethylene vinyl acetate copolymer or a fibrin

matrix layer on a portion of the ethylene vinyl acetate copolymer interior surface as required by Claims 1, 48 and 51.

Turner is directed to a system for selectively immobilizing stem cells that comprises combining a substrate having a coating comprising a fibrin matrix together with a substance capable of binding to the fibrin matrix and having a binding site for binding an RGD amino acid sequence for binding to the stem cells. See *Turner*, column 2, lines 35-50. *Turner* fails to disclose or suggest a flexible interior surface comprising an ethylene vinyl acetate copolymer or a fibrin matrix layer on a portion of the ethylene vinyl acetate copolymer interior surface as required by Claims 1, 48 and 51. In fact, *Turner* fails to even disclose or suggest the use of an ethylene vinyl acetate copolymer anywhere in his disclosure.

Codner is directed to cell culture apparatus formed of a plurality of thin (e.g., 0.005" or less), spaced, gas-permeable, silicone membranes sealed at their edges to form a bag-like vessel comprising one or more interior chambers suitable for containing cell culture media. See *Codner*, column 2, lines 66 to column 3, line 8. A suitable portion of the membrane surfaces are of suitable thickness and surface area to provide structural integrity to the apparatus and sufficient gas permeability for cell growth within the chamber. Nevertheless, *Codner* fails to disclose or suggest a fibrin matrix layer on a portion of an ethylene vinyl acetate copolymer interior surface as required by Claims 1, 48 and 51. In fact, *Codner* fails to even disclose or suggest the use of fibrin for growing cells anywhere in his disclosure.

For at least the reasons discussed above, *Smith*, *Toner*, *Turner* and *Codner* alone or in combination fail to disclose or suggest each and every element of independents Claims 1, 48 and 51. Accordingly, Appellants respectfully submit that Claims 1, 48 and 51, as well as Claims 3-11, 19-21, 23-47, 49-50 and 52-53 that depend from Claims 1, 48 and 51, are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

2. The skilled artisan would have no reason to combine *Smith*, *Toner*, *Turner* and *Codner* to arrive at the claimed invention

Appellants respectfully submit that the skilled artisan would have no reason to combine the cited references in the absence of hindsight because the cited references teach away from

each other and/or the claimed invention. Moreover, Appellants respectfully submit that the Examiner is using Appellants' patent application as a road map for creating hindsight obviousness and has failed to set forth sufficient reasons for how the skilled artisan would have arrived at the claimed invention in view of *Smith*, *Toner*, *Turner* and *Codner*.

Smith is entirely directed to a multi-layer, flexible, gas-permeable container. See *Smith*, column 2, lines 24-32. The outer walls that make up the container of *Smith* are made of the flexible, gas-permeable materials. *Smith* emphasizes the advantages of the container being gas-permeable to culture cells. For example, *Smith* teaches that it has been found that the cell growth rate within a sealed container may be influenced by the gas permeability characteristics of the container walls. See *Smith*, column 2, lines 13-20 and 52-65. As a result, *Smith* provides a flexible, gas-permeable cell culture container whose gas permeability may be adjusted to best match the requirements of the cell being cultured in the container.

Smith further teaches the problems associated with using containers having rigid and impermeable walls for cultured cell growth. *Smith* teaches that the known types of rigid cell culture containers are gas impermeable and have lids or caps that are not sealed onto the containers. See *Smith*, column 1, lines 46-62. They are offset sufficiently to allow gas exchange through a gap or vent between the cap and the container. *Smith* explicitly states that such a container is disadvantageous for clinical uses because the vent might allow contamination of the culture or lead to accidents involving biohazardous agents.

Toner teaches away from a combination with *Smith* by teaching a device having completely a different mode of operation than *Toner*. *Toner* is entirely directed to a modular cell culturing device including one or more two-compartment cartridge entirely comprising rigid and impermeable exterior walls 50. See *Toner*, column 2, lines 39-45, column 7, lines 38-59, column 11, lines 27-41 ("rigid impermeable walls 50"). The rigid walls of the cartridges in *Toner* are specifically intended and designed for being impermeable to liquids and gases to adequately maintain the bioreactor. See *Toner*, column 7, lines 54-63. As a result, *Toner's* disclosure explicitly teaches away from *Smith's* flexible, gas-permeable container, which would lead the skill artisan away from combining *Smith* and *Toner*.

The Examiner uses *Toner* merely for the fact that *Toner* discloses fibrin in a cell culture device (albeit completely different cell culture device from that of Smith). *Toner* teaches a polymeric membrane 30 (which may be coated with fibrin) that separates a liquid compartment

and an oxygenated fluid compartment of the cartridge. Nevertheless, *Toner* teaching using fibrin as a coating matter, which is already known in the art. Ignoring other teaching away aspects of *Toner* is a strong indication that the Examiner is using Appellants' disclosure as a blueprint to pick and choose from isolated portions of the prior art in order to deprecate Appellants' claims. Such conduct is exemplary of hindsight reasoning, which is clearly improper.

Moreover, *Toner's* use of fibrin is specific to his device. For example, *Toner* does not coat the rigid, impermeable walls of the cartridges, but the intermediate separating membrane within the cartridge. As a result, the cells growth takes place on the intermediate membrane and not on the interior of the outer cartridge walls.

If the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, then the teachings of the references are not sufficient to render the claims prima facie obvious. *In re Ratti*, 270 F.2d 810, 123 USPQ 349 (CCPA 1959). This certainly applies here where one of the cited references is directed to a container that functions because it is flexible and gas-permeable (*Smith*) and the other cited reference is directed to a cartridge that functions based on having rigid and impermeable exterior walls (*Toner*). As a result, the principle of operation for each device is important and specific to that particular device. The Examiner has provided no evidence that the device of *Smith* works as modified by *Toner* and vice versa and has failed to consider the portions of the references that teach away from the combination. Because of these differences, one skilled in the art would have no reason to modify or combine *Toner* and *Smith* to arrive at the present claims.

The skilled artisan would also have no reason to use *Smith* to arrive at the present claims because *Smith* teaches away from the present claims. For example, *Smith* explicitly teaches away from an interior surface comprising an ethylene vinyl acetate by stating:

> While EVA [ethylene vinyl acetate] can hold an electrostatic charge, the charge has the undesirable tendency to decay over time. Eventually, the decay of the charge on EVA will render the container ineffective for growing adherent cells. Rigid styrene flasks with an electrostatic charge are known, and show less of a tendency to lose charge over time.
>
> See *Smith*, column 2, lines 7-12 (emphasis added).

Accordingly, *Smith* teaches away from using any ethylene vinyl acetate copolymer for an interior surface of a cell culture container in accordance with the present claims.

Toner also explicitly teaches away from the present claims. For example, *Toner* is entirely directed to an open or flow-through cell-culturing device (see *Toner*, column 2, lines 35-50), which teaches away from a closed container in accordance with the present claims. *Toner's* cartridge includes oxygenated fluid inlet/out 3,3' and liquid inlet/outlet 5,5'. The inlets and outlets permit continuous fluid flow through *Toner's* cartridge. The inlets and outlets fulfill an objective of *Toner*, which is to cultivate cells on membrane 30 by passing flowing fluid along each side of the membrane 30. See *Toner*, column 2, lines 35-45. Accordingly, *Toner's* flow-through cartridge is an open system, which leads the skilled artisan away from a closed supporting container in accordance with the present claims.

The skilled artisan would have no reason to combine *Turner* and *Codner* with *Smith* and *Toner* to arrive at the present claims. *Turner* fails to even disclose or suggest the use of and ethylene vinyl acetate copolymer anywhere in his disclosure. *Codner* fails to even disclose or suggest the use of fibrin for growing cells anywhere in his disclosure, especially in a container having an inner layer of an ethylene vinyl acetate copolymer.

Appellants respectfully submit that the Examiner has failed to set forth sufficient reasons for combining the cited references in the absence of hindsight. Rather, the Examiner generally concludes that it would have been within the ordinary skill of the art at the time the claimed invention to combine the references because the references relied upon allegedly teach that all aspects of the claimed invention were individually known in the art. However, this conclusory statement is not sufficient to establish a *prima facie* case of obviousness without some objective reason to combine the teachings of the references. *Ex parte Levengood*, 28 USPQ2d 1300 (Bd. Pat. App. & Inter. 1993). There must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness by the Examiner. *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006).

In sum, the skilled artisan would have no reason to combine *Smith*, *Toner*, *Turner* and *Codner* because the cited references have different modes of operation and teach away from each other and/or the present claims. Moreover, the cited references fail to even recognize the advantages, benefits and/or properties of a fibrin matrix layer on a portion of an interior surface composed of an ethylene vinyl acetate copolymer in accordance with the present claims and

provide no reasonable expectation of success with respect to same. Instead, Appellants respectfully submit that the Examiner is improperly using Appellants' patent application as a road map for creating hindsight obviousness. Accordingly, Appellants respectfully submit that independent Claims 1, 48 and 51, along with Claims 3-11, 19-21, 23-47, 49-50 and 52-53 that depend from Claims 1, 48 and 51, are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

D. THE REJECTION OF CLAIMS 36-46 UNDER 35 U.S.C. §103(A) TO *SMITH, TONER, TURNER, CODNER* AND *DELMOTTE* IS IMPROPER IN VIEW OF THE PATENTABILITY OF INDEPENDENT CLAIM 1

Claims 36-46 stand rejected under 35 U.S.C. §103(a) as being unpatentable over *Smith*, *Toner*, *Turner, Codner* and *Delmotte*. Appellants respectfully submit that the patentability of Claim 1 over *Smith*, *Toner*, *Turner* and *Codner* as discussed above also demonstrates that the obviousness rejection of Claims 36-46, which depend from Claim 1, is improper. In this regard, even with *Delmotte* as a reference, the cited art fails to teach or suggest the elements of Claims 36-46 in combination with the novel elements of Claim 1.

For example, *Smith*, *Toner*, *Turner* and *Codner* fail to disclose or suggest a number of elements required by Claim 1 as discussed previously. *Smith*, *Toner* and *Turner* fail to disclose or suggest a flexible interior surface comprising an ethylene vinyl acetate copolymer as required by Claim 1. *Smith*, *Toner*, *Turner* and *Codner* also fail to disclose or suggest a fibrin matrix layer on a portion of the ethylene vinyl acetate copolymer interior surface as required by Claim 1.

Delmotte fails to remedy the deficiencies of *Smith*, *Toner*, *Turner* and *Codner*. *Delmotte* fails to disclose or suggest an interior surface comprising an ethylene vinyl acetate copolymer as required, in part, by Claims 1, 48 and 51. *Delmotte* also fails to disclose or suggest a fibrin matrix layer on a portion of the ethylene vinyl acetate copolymer interior surface as required by Claim 1. Instead, *Delmotte* is directed to rigid syringes that are used as a medical delivery device. *Delmotte* fails to even disclose or suggest the use of ethylene vinyl acetate copolymer anywhere in his disclosure.

In addition, *Delmotte* teaches away from the present claims and a combination with *Smith*. *Delmotte* discloses a fibrin delivery device 10 having first and second syringes 12, 14 and a spray unit 18. A pressurizer 22 travels through each syringe 12, 14 to push fluid present in each syringe through the spray unit 18. *Delmotte*, column 8, lines 31-43, column 9, lines 47-58, Figures 1 and 4. One of ordinary skill in the art would recognize that syringes 12, 14 are rigid and impermeable in order to withstand the pressure imposed by pressurizer 22 when pushing fluid out of each syringe. Accordingly, *Delmotte* teaches away from a closed support container having flexible and gas permeable exterior sidewalls in accordance with the present claims. Moreover, *Delmotte's* rigid, impermeable syringes teach away from the flexible, permeable container of *Smith*.

Accordingly, Appellants respectfully submit that Claims 36-46 are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

VIII. CONCLUSION

Appellants respectfully submit that the Examiner has failed to establish a *prima facie* case of obviousness under 35 U.S.C. §103 with respect to the rejections of Claims 1, 3-11, 19-21 and 23-53. Accordingly, Appellants respectfully submit that the obviousness rejections are erroneous in law and in fact and should therefore be reversed by this Board.

A check in the amount of $510 is submitted herewith to cover the cost of the Appeal Brief. The Director is authorized to charge any additional fees that may be required, or to credit any overpayment to Deposit Account No. 02-1818. If such a withdrawal is made, please indicate the Attorney Docket No. 112713-983 on the account statement.

Respectfully submitted,

BELL, BOYD & LLOYD LLC

BY 

Robert M. Barrett
Reg. No. 30,142
Customer No. 29200
Phone No. 312-807-4204

Dated: October 8, 2008

CLAIMS APPENDIX

PENDING CLAIMS ON APPEAL OF U.S. PATENT APPLICATION SERIAL NO. 10/634,663

1. A cell culture container comprising:

a closed supporting container comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area, the first side wall being constructed from a gas permeable material selected from the group consisting of polymeric material, paper, and fabric, the first side wall having a gas permeability sufficient to permit cellular respiration, and the second side wall being constructed from a material selected from the group consisting of polymeric material, paper, fabric, and foil, wherein at least one of the first and the second side wall comprises an interior surface comprising an ethylene vinyl acetate copolymer; and

a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container.

3. The cell culture container of claim 1 wherein the polymeric material of the first side wall or the second side wall of the supporting container is a multiple-component polymer blend.

4. The cell culture container of claim 3 wherein at least one of the components of the multiple-component polymer blend is a styrene and hydrocarbon copolymer.

5. The cell culture container of claim 1 wherein the gas permeable material is a monolayer structure.

6. The cell culture container of claim 1 wherein the gas permeable material is a multilayer structure.

7. The cell culture container of claim 6 wherein the multilayer structure comprises: a first layer comprising a first ethylene vinyl acetate copolymer, the first layer having a first surface and a second surface; and a second layer adhering to the first surface of the first layer, the second layer comprising a second ethylene vinyl acetate copolymer; wherein the second surface of the first layer forms the interior surface of the supporting container.

8. The cell culture container of claim 7 wherein the first ethylene vinyl acetate copolymer having a vinyl acetate content of greater than 18% by weight of the copolymer.

9. The cell culture container of claim 7 wherein the second ethylene vinyl acetate copolymer having a vinyl acetate content of less than 18% by weight of the copolymer.

10. The cell culture container of claim 7 wherein the first ethylene vinyl acetate copolymer having a vinyl acetate content of about 18% by weight of the copolymer.

11. The cell culture container of claim 7 wherein the second ethylene vinyl acetate copolymer having a vinyl acetate content of about 9% by weight of the copolymer.

19. The cell culture container of claim 1, wherein the second side wall comprises a gas permeable material that is a monolayer structure.

20. The cell culture container of claim 1, wherein the second side wall comprises a gas permeable material that is a multilayer structure.

21. The cell culture container of claim 20, wherein the multilayer structure comprises: a first layer comprising first ethylene vinyl acetate copolymer with a vinyl acetate content of greater than 18% by weight of the copolymer, the first layer having a first surface and a second surface; and a second layer adhering to the first surface of the first layer, the second layer comprising a second ethylene vinyl acetate copolymer with a vinyl acetate content of from less than 18% by weight of the copolymer, wherein the second surface of the first layer forms the inner surface of the container.

23. The cell culture container of claim 21 wherein the vinyl acetate content of the second vinyl acetate copolymer in the second layer is about 9% by weight of the copolymer.

24. The cell culture container of claim 20, wherein the multilayer structure comprises: a first layer comprising polystyrene having a thickness within the range of 0.0001 inches to about 0.0010 inches; and a second layer adhering to the first layer, the second layer comprising a polymeric material selected from the group consisting of ethylene vinyl acetate copolymers, polyolefins, polyamides, polyesters, styrene and hydrocarbon copolymers, fluorocarbon elastomers, the second layer having a thickness within the range of 0.004 inches to about 0.025 inches.

25. The cell culture container of claim 24, wherein the polymeric material of the second layer is a multi-component polymer blend.

26. The cell culture container of claim 25, wherein at least one of the components of the multi-component polymer blend is a styrene and hydrocarbon copolymer.

27. The cell culture container of claim 1, wherein the container has an oxygen permeability of from about 9 to about 15 Barrers, a carbon dioxide permeability of from about 40 to about 80 Barrers, a nitrogen permeability of from about 10 to about 100 Barrers, and a water vapor transmission rate of less than about 20 (g mil/100 in^2/day).

28. The cell culture container of claim 1, wherein the first side wall and the second side wall have a flexural modulus of from about 10,000 psi to about 30,000 psi as measured according to ASTM D-790.

29. The cell culture container of claim 1, wherein at least a portion of the container is optically clear.

30. The cell culture container of claim 1, wherein a substantial portion of the container is optically clear.

31. The cell culture container of claim 1, wherein the container is radiation sterilizable.

32. The cell culture container of claim 1, wherein the container further comprises at least one port providing access to the containment area.

33. The cell culture container of claim 1, wherein the fibrin matrix extends over substantially an entire surface of the interior surface of at least one of the side walls.

34. The cell culture container of claim 1, wherein the fibrin matrix is on at least a portion of the interior surface of each of the side walls.

35. The cell culture container of claim 1, wherein the fibrin matrix is three-dimensional with pore sizes of from about 0.5 to about 5.0 um in diameter.

36. The cell culture container of claim 1, wherein the fibrin matrix is formed by cross-linking fibrin or fibrinogen.

37. The cell culture container of claim 1, wherein the fibrin matrix is prepared by mixing a first solution comprising fibrinogen and factor XIII with a second solution comprising thrombin and calcium to form the fibrin matrix.

38. The cell culture container of claim 37, wherein the fibrinogen is derived from mammalian plasma.

39. The cell culture container of claim 38, wherein the mammalian plasma is human plasma.

40. The cell culture container of claim 37, wherein the fibrinogen is a recombinant fibrinogen.

41. The cell culture container of claim 37, wherein the factor XIII is derived from mammalian plasma.

42. The cell culture container of claim 41, wherein the mammalian plasma is human plasma.

43. The cell culture container of claim 37, wherein the factor XIII is a recombinant factor XIII.

44. The cell culture container of claim 37, wherein the thrombin is derived from mammalian plasma.

45. The cell culture container of claim 44, wherein the mammalian plasma is selected from the group consisting of bovine plasma and human plasma.

46. The cell culture container of claim 37, wherein the thrombin is a recombinant thrombin.

47. The cell culture container of claim 37 wherein the concentration of fibrinogen in the first solution is from about 2.0 to about 20 mg/mL, the concentration of the factor XIII in the first solution is from about 10 to about 40 IU/mL, the concentration of the thrombin in the second solution is from about 2.5 IU/mL to about 50 IU/mL, and the concentration of the calcium in the second solution is from about 40 to about 100 mmoles/mL, about 0.5-1.0 mLs of the first solution is mixed with 0.5-1.0 mLs of the second solution to form a fibrin-forming mixture.

48. A cell culture container comprising:

a closed supporting container comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area, each side wall having an interior surface, the first side wall and the second side wall comprising an interior surface constructed from an ethylene vinyl acetate copolymer having a gas permeability sufficient to permit cellular respiration; and

a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container.

49. The cell culture container of claim 48, wherein the ethylene vinyl acetate copolymer is a multilayer structure comprising: a first layer comprising a first ethylene vinyl acetate copolymer with a vinyl acetate content of greater than 18% by weight of the copolymer, the first layer having a first surface and a second surface; and a second layer adhering to the first surface of the first layer, the second layer comprising a second ethylene vinyl acetate copolymer with a vinyl acetate content of less than 18% by weight of the copolymer; wherein the second surface of the first layer forms the interior surface of the supporting container.

50. The cell culture container of claim 48, wherein the vinyl acetate content of the second ethylene vinyl acetate copolymer is about 9% by weight of the copolymer.

51. A cell culture container comprising:

a closed supporting container comprising a first flexible exterior side wall connected to a portion of an opposing second flexible exterior side wall along a peripheral seal to define a containment area, the side walls are constructed from a multilayer gas permeable polymeric structure having a gas permeability sufficient to permit cellular respiration, and the multilayer polymeric structure comprising:

a first layer comprising polystyrene having a thickness within the range of 0.0001 inches to about 0.0010 inches; and

a second layer adhering to the first layer, the second layer comprising an interior surface comprised of an ethylene vinyl acetate copolymer, the second layer having a thickness within the range of 0.004 inches to about 0.025 inches; and

a fibrin matrix layer on a portion of the interior surface of the first side wall or the second side wall of the supporting container.

52. The cell culture container of claim 51, wherein the polymeric material of the second layer is a multi-component polymer blend.

53. The cell culture container of claim 52, wherein at least one of the components of the multi-component polymer blend is a styrene and hydrocarbon copolymer.

EVIDENCE APPENDIX

EXHIBIT A: Non-final Office Action dated November 26, 2007

EXHIBIT B: Final Office Action dated June 5, 2008

EXHIBIT C: U.S. Patent No. 5,935,847 to Smith et al. ("*Smith*"), cited by the Examiner in the Office Action dated June 5, 2008

EXHIBIT D: U.S. Patent No. 6,759,245 to Toner et al. ("*Toner*"), cited by the Examiner in the Office Action dated June 5, 2008

EXHIBIT E: U.S. Patent No. 5,912,177 to Turner ("*Turner*"), cited by the Examiner in the Office Action dated June 5, 2008

EXHIBIT F: U.S. Patent No. 5,686,304 to Codner ("*Codner*"), cited by the Examiner in the Office Action dated June 5, 2008

EXHIBIT G: U.S. Patent No. 5,989,215 to Delmotte ("*Delmotte*"), cited by the Examiner in the Office Action dated June 5, 2008

RELATED PROCEEDINGS APPENDIX

None

EXHIBIT A

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United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/634,663	08/05/2003	Sidney T. Smith	TR-5934	6356

29200 7590 11/26/2007

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ART UNIT	PAPER NUMBER
1797	

MAIL DATE	DELIVERY MODE
11/26/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

RECEIVED
BELL, BOYD & LLOYD
INTELLECTUAL PROPERTY DOCKET
JAN 04 2008
ATTY:
DOCKET #:

DOCKETED
DEC 18 2007

Office Action Summary	Application No.	Applicant(s)	
	10/634,663	SMITH ET AL.	
	Examiner	Art Unit	
	Nathan A. Bowers	1797	

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *21 September 2007*.

2a)☐ This action is FINAL. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-11,19-21 and 23-53* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1, 2-11, 19-21 and 23-5* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1 ☐ Certified copies of the priority documents have been received.

2 ☐ Certified copies of the priority documents have been received in Application No. _____.

3 ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 21 September 2007 has been entered.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any

evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

1) Claims 1, 3-11, 19-21, 23-34 and 48-53 are rejected under 35 U.S.C. 103(a) as being unpatentable over Smith (US 5935847) in view of either Toner (US 6759245) or Turner (US 5912177), and further in view of Codner (US 5686304).

With respect to claims 1 and 48, Smith discloses a closed cell culture container (Figure 2:20) comprising a first flexible sidewall (Figure 2:22) connected to a portion of an opposing second flexible sidewall (Figure 2:24) along a peripheral seal to define a containment area (Figure 2:26). This is disclosed in column 6, lines 12-33. Column 2, lines 24-31 and column 3, line 59 to column 4, line 46 teach that the first and second sidewalls are constructed from flexible polymeric materials that permit cellular respiration. Column 5, lines 39-45 indicate that the sidewalls are constructed from ethylene vinyl acetate. A polystyrene layer (Figure 2:12) is provided to promote cell adhesion to the inside surface of the culture container. Smith, however, does not expressly state that a fibrin matrix layer is positioned on a portion of the interior surface of the first or second sidewalls of the cell culture container.

Toner discloses a cell culturing device (Figure 1) that includes a chamber divided by a gas permeable, liquid impermeable polymeric membrane (Figure 2:30). Cells (Figure 2:40) are seeded upon the membrane, and gases from an oxygenated liquid stream (Figure 2:20) are allowed to diffuse through the membrane in order to contact the cells. This is disclosed in

column 3, lines 1-25 and column 7, line 10 to column 8, line 19. Column 9, lines 8-42 indicate that the membrane may be constructed from a variety of polymer compounds arranged in a single or multi-layered assembly. Column 11, lines 27-56 teach that the membrane (Figure 1:30) is coated with a fibrin matrix layer (Figure 1:41) to increase cell adhesion.

Turner discloses a polymer bag that forms a closed container for holding a cell culture. Column 3, lines 47-65 state that the bag is permeable to gases vital for cellular metabolism. Column 2, lines 43-50 indicate that a fibrin matrix is immobilized upon the inner walls of the bag in order to facilitate the adhesion of cells.

Smith, Toner and Turner are analogous art because they are from the same field of endeavor regarding cell culture containers.

At the time of the invention, it would have been obvious to include a fibrin matrix layer positioned on the interior surface of the polystyrene layer disclosed by Smith. In column 6, line 65 to column 7, line 19, Smith teaches that it is desirable to provide a culture vessel which includes sidewalls that are capable of accommodating adherent dependent cells. Toner and Turner each teach that fibrin, when applied to a polymer substrate, will enhance cell immobilization to the polymer substrate. In this way, Smith's invention would be improved through the addition of a fibrin matrix layer because the fibrin matrix would allow the cell culture container to better accommodate a wider range of adherent dependent cell types.

The combination of Smith and Toner/Turner still differs from Applicant's claimed invention because it is not entirely clear if the inner surface of Smith's container comprises an ethylene vinyl acetate copolymer. The Figures predominantly indicate that the inner surface of

the container is covered by a polystyrene layer (Figure 8:28) as opposed to an ethylene vinyl acetate copolymer layer (Figure 8:24).

Codner discloses a cell culture apparatus. In column 6, line 53 to column 7, line 5, Codner teaches that the walls defining the apparatus comprise an inner surface formed from an ethylene vinyl acetate copolymer.

Smith, Toner, Turner and Codner are analogous art because they are from the same field of endeavor regarding cell culture bags.

At the time of the invention, it would have been obvious to form the inner surfaces of Smith's container from areas comprising polystyrene and fibrin materials as well as areas comprising ethylene vinyl acetate copolymers. As previously described above, polystyrene and fibrin are beneficial because they foster the growth of adherent cells. However, Smith teaches in column 7, lines 35-36 that ethylene vinyl acetate is more suitable for the culture of non-adherent cells, and, as evidenced by Codner, the use of ethylene vinyl acetate copolymers is well known in the art. It would have been obvious to ensure that some areas of Smith's inner surface are covered by fibrin and polystyrene to encourage the growth of adherent cells, and it would have been obvious to ensure that other areas of Smith's inner surface are covered by ethylene vinyl acetate copolymers to promote the culture of non-adherent cells.

With respect to claims 3 and 4, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith teaches in column 4, lines 11-46 that the gas permeable material is either EVA, polyolefin, polyamide or styrene. The

polymeric material of the first sidewall is a styrene and hydrocarbon multi-component polymer blend.

With respect to claims 5-11, 49 and 50, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith teaches that the gas permeable material is either a monolayer or a multilayer structure. Monolayer cell culture containers are well known in the art, and Figures 1 and 4 illustrate multilayer embodiments. A polystyrene layer (Figure 4:12) and a skin layer (Figure 4:18) are provided in addition to the substrate layer (Figure 4:14). Column 5, lines 7-18 teach that the skin layer and substrate layer are formed on the outer surface of the polystyrene layer, so that the inner surface of the polystyrene layer forms the interior surface of the culture chamber. The skin layer is formed from polyethylene copolymers and polypropylene copolymers. Column 4, lines 11-46 indicate that substrate layer is anywhere from 0-40% ethylene vinyl acetate copolymer. It is an intrinsic feature of the invention that the composition of the substrate and polystyrene layers can be manipulated in order to achieve any desired polymer distribution.

The claimed weight ratios are simply result effective variables. In the absence of new or unexpected results, it would have been obvious to optimize the composition of the substrate and skin layers. This optimization could simply be accomplished by producing different compositions and testing their ability to be used in cell culturing. See *In re Boesch*, 617 F.2d 272, 205 USPQ 215 (CCPA 1980).

With respect to claims 19-21, 23-26 and 51-53, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith states in column 2, lines 24-31 that the polystyrene layer (1st layer) has a thickness within the range of 0.0001 inches to 0.001 inches. Column 4, lines 47-56 indicate that the substrate layer (2nd layer) has a thickness of 0.004 inches to 0.025 inches. Column 4, lines 11-46 teach that the second layer is a multi-component polymer blend that includes styrene and hydrocarbon copolymer. Figure 2 indicates that the gas permeable EVA material is used in the construction of both the first and second sidewalls. The nature of the invention regarding copolymer content and layer thickness has already been described.

With respect to claims 27-32, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith states in column 2, lines 39-51 that the culture container has a oxygen permeability of 9-15 Barrers, a carbon dioxide permeability of 40-80 Barrers, a nitrogen permeability of 10-100 Barrers and a water vapor transmission rate of less than 20 (g mil/100 in^2/day). Column 5, line 49 to column 6, line 8 indicates that the first and second sidewalls have a flexural modulus of 10,000-30,000 psi, and that the sidewalls are optically clear. The container is radiation sterilizable. Column 7, lines 39-44 indicate that at least one port (Figure 9:40) provides access to the containment area.

With respect to claims 34 and 35, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith states in column 6, line 66 to column 7, line 19 that the inside surfaces of the culture container can be modified in order to

determine to what areas cells are allowed to adhere. Accordingly, it would have been obvious to apply the fibrin matrix disclosed by Toner to any part of the container surface that is desired to promote cell adhesion. This intrinsically could pertain to the entire inner surface of the container, or just specific regions of the inner surface. If the culture container is intended to facilitate the growth of adherent cell types, then it would be obvious to apply the fibrin matrix to the entire sidewall interior surface. If the culture container is intended to facilitate the growth of adherent and non-adherent cell types, then it would be obvious to apply to fibrin matrix to just a part of the sidewall interior surface.

2) Claims 36-46 are rejected under 35 U.S.C. 103(a) as being unpatentable over Smith (US 5935847) in view of Toner (US 6759245)/Turner (US 5912177) and Codner (US 5686304) as applied to claim 1, and further in view of Delmotte (US 5989215).

With respect to claims 35-37, Smith, Toner/Turner and Codner disclose the invention set forth in the 35 U.S.C. 103 rejections above, however do not expressly disclose the nature of the fibrin matrix.

Delmotte discloses a method for forming a fibrin matrix that includes delivering a first solution of fibrinogen and factor XIII and a second solution of thrombin and calcium to a desired surface. This is disclosed in column 3, lines 31-44 and column 8, lines 3-15. In column 12, line 34 to column 13, line 20, Delmotte states that the amount of thrombin added to the fibrinogen solution is directly related to the pore size of the fibrin matrix product. Thrombin can be added in varying amounts in order to create a fibrin network characterized by pore diameters anywhere between 0.2-4 microns.

Smith, Toner/Turner, Codner and Delmotte are analogous art because they are from the same field of endeavor regarding cell culture systems.

At the time of the invention, it would have been obvious to form a fibrin matrix within the cell culture container disclosed by Smith and Toner by mixing a solution of fibrinogen with a solution of thrombin. In column 4, line 57 to column 5, line 16, Delmotte states that by separating fibrinogen and thrombin into two separate solutions, one is able to more easily manipulate the concentrations of fibrinogen and thrombin to effect change in the characteristics of the resultant fibrin film. In this way, the concentration of thrombin can be readily changed in order to create a fibrin matrix with a desired pore size.

With respect to claims 38-46, Smith, Toner/Turner, Codner and Delmotte disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In column 7, lines 29-32, Delmotte teaches that the components of the fibrinogen and thrombin are derived from human plasma. It would have been obvious to utilize recombinant components of fibrinogen and thrombin, as well. When the fibrin matrix is used in a bioreactor and not for treating a human being, it is less important to use fibrinogen and thrombin attained from human blood plasma. Techniques for creating recombinant biomolecules are well known in the art.

With respect to claim 47, Smith, Toner/Turner, Codner and Delmotte disclose the apparatus set forth in claim 37 as set forth in the 35 U.S.C. 103 rejection above. In addition, Delmotte discloses in column 8, lines 3-29 that fibrin is made from a first solution containing 10-40 IU/ml of fibrinogen and factor XIII, and a second solution containing 3-10,000 IU/ml of

thrombin and 45 micromoles/ml of calcium. Column 15, lines 1-15 disclose a method in which the fibrinogen and thrombin solutions are repeatedly applied to a surface in 0.3 ml increments. Column 18, lines 51-63 disclose a method in which 3.5 ml of the fibrinogen and thrombin solutions are mixed to form a fibrin matrix. The fibrinogen and thrombin solutions are incubated, and the formed fibrin matrix has a pore size of anywhere between 0.2-4 microns.

Response to Arguments

Applicant's arguments filed 21 September 2007 with respect to the 35 U.S.C. 103 rejections involving the combination of Smith and Toner have been fully considered and are persuasive. Therefore, these rejections have been withdrawn. However, upon further consideration, a new ground of rejection is made in view of the combination of Smith with either Toner or Turner and further in view of Codner.

The Turner reference clearly indicates that it is known in the art to coat the interior of a bag culture container with a fibrin matrix.

The Codner reference teaches that it is known in the art to construct a bag culture container such that the interior surface is formed from an ethylene vinyl acetate copolymer. Smith teaches in column 7, lines 35-36 that ethylene vinyl acetate is advantageous for the culture of non-adherent cells.

Conclusion

This is a non-final rejection.

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Nathan A. Bowers whose telephone number is (571) 272-8613. The examiner can normally be reached on Monday-Friday 8 AM to 5 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gladys Corcoran can be reached on (571) 272-1214. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

NAB

GLADYS JP CORCORAN
SUPERVISORY PATENT EXAMINER

Notice of References Cited	Application/Control No. 10/634,663	Applicant(s)/Patent Under Reexamination SMITH ET AL.	
	Examiner Nathan A. Bowers	Art Unit 1797	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,912,177	06-1999	Turner et al.	435/455
*	B	US-5,686,304	11-1997	Codner, Meryl	435/325
	C	US-			
	D	US			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EXHIBIT B

5934 (RJ) US

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/634,663	08/05/2003	Sidney T. Smith	TR-5934	6356

28200 7590 06/05/2008
BAXTER HEALTHCARE CORPORATION
1 BAXTER PARKWAY
DP2-2E
DEERFIELD, IL 60015

EXAMINER
BOWERS, NATHAN ANDREW

ART UNIT	PAPER NUMBER
1797	

MAIL DATE	DELIVERY MODE
06/05/2008	PAPER

AO. 9-5-08: FINAL

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

RECEIVED

JUN 09 2008

Corporate Patent Administration

RECEIVED
BELL, BOYD & LLOYD
INTELLECTUAL PROPERTY DOCKET

JUN 12 2008

ATTY: KMB MJB
DOCKET #: 112713-

983

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No.	Applicant(s)	
	10/634,663	SMITH ET AL.	
	Examiner	Art Unit	
	NATHAN A. BOWERS	1797	

– The MAILING DATE of this communication appears on the cover sheet with the correspondence address –

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *26 February 2008*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,3-11,19-21 and 23-53* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,3-11,19-21 and 23-53* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

1) Claims 1, 3-11, 19-21, 23-34 and 48-53 are rejected under 35 U.S.C. 103(a) as being unpatentable over Smith (US 5935847) in view of either Toner (US 6759245) or Turner (US 5912177), and further in view of Codner (US 5686304).

With respect to claims 1 and 48, Smith discloses a closed cell culture container (Figure 2:20) comprising a first flexible sidewall (Figure 2:22) connected to a portion of an opposing second flexible sidewall (Figure 2:24) along a peripheral seal to define a containment area (Figure 2:26). This is disclosed in column 6, lines 12-33. Column 2, lines 24-31 and column 3, line 59 to column 4, line 46 teach that the first and second sidewalls are constructed from flexible polymeric materials that permit cellular respiration. Column 5, lines 39-45 indicate that the sidewalls are constructed from ethylene vinyl acetate. A polystyrene layer (Figure 2:12) is provided to promote cell adhesion to the inside surface of the culture container. Smith, however, does not expressly state that a fibrin matrix layer is positioned on a portion of the interior surface of the first or second sidewalls of the cell culture container.

Toner discloses a cell culturing device (Figure 1) that includes a chamber divided by a gas permeable, liquid impermeable polymeric membrane (Figure 2:30). Cells (Figure 2:40) are seeded upon the membrane, and gases from an oxygenated liquid stream (Figure 2:20) are allowed to diffuse through the membrane in order to contact the cells. This is disclosed in column 3, lines 1-25 and column 7, line 10 to column 8, line 19. Column 9, lines 8-42 indicate that the membrane may be constructed from a variety of polymer compounds arranged in a single or multi-layered assembly. Column 11, lines 27-56 teach that the membrane (Figure 1:30) is coated with a fibrin matrix layer (Figure 1:41) to increase cell adhesion.

Turner discloses a polymer bag that forms a closed container for holding a cell culture. Column 3, lines 47-65 state that the bag is permeable to gases vital for cellular metabolism. Column 2, lines 43-50 indicate that a fibrin matrix is immobilized upon the inner walls of the bag in order to facilitate the adhesion of cells.

Smith, Toner and Turner are analogous art because they are from the same field of endeavor regarding cell culture containers.

At the time of the invention, it would have been obvious to include a fibrin matrix layer positioned on the interior surface of the polystyrene layer disclosed by Smith. In column 6, line 65 to column 7, line 19, Smith teaches that it is desirable to provide a culture vessel which includes sidewalls that are capable of accommodating adherent dependent cells. Toner and Turner each teach that fibrin, when applied to a polymer substrate, will enhance cell immobilization to the polymer substrate. In this way, Smith's invention would be improved through the addition of a fibrin matrix layer because the fibrin matrix would allow the cell culture container to better accommodate a wider range of adherent dependent cell types.

The combination of Smith and Toner/Turner still differs from Applicant's claimed invention because it is not entirely clear if the inner surface of Smith's container comprises an ethylene vinyl acetate copolymer. The Figures predominantly indicate that the inner surface of the container is covered by a polystyrene layer (Figure 8:28) as opposed to an ethylene vinyl acetate copolymer layer (Figure 8:24).

Codner discloses a cell culture apparatus. In column 6, line 53 to column 7, line 5, Codner teaches that the walls defining the apparatus comprise an inner surface formed from an ethylene vinyl acetate copolymer.

Smith, Toner, Turner and Codner are analogous art because they are from the same field of endeavor regarding cell culture bags.

At the time of the invention, it would have been obvious to form the inner surfaces of Smith's container from areas comprising polystyrene and fibrin materials as well as areas comprising ethylene vinyl acetate copolymers. As previously described above, polystyrene and fibrin are beneficial because they foster the growth of adherent cells. However, Smith teaches in column 7, lines 35-36 that ethylene vinyl acetate is more suitable for the culture of non-adherent cells, and, as evidenced by Codner, the use of ethylene vinyl acetate copolymers is well known in the art. It would have been obvious to ensure that some areas of Smith's inner surface are covered by fibrin and polystyrene to encourage the growth of adherent cells, and it would have been obvious to ensure that other areas of Smith's inner surface are covered by ethylene vinyl acetate copolymers to promote the culture of non-adherent cells.

With respect to claims 3 and 4, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith teaches in column 4, lines 11-46 that the gas permeable material is either EVA, polyolefin, polyamide or styrene. The polymeric material of the first sidewall is a styrene and hydrocarbon multi-component polymer blend.

With respect to claims 5-11, 49 and 50, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith teaches that the gas permeable material is either a monolayer or a multilayer structure. Monolayer cell culture containers are well known in the art, and Figures 1 and 4 illustrate multilayer embodiments. A polystyrene layer (Figure 4:12) and a skin layer (Figure 4:18) are provided in addition to the substrate layer (Figure 4:14). Column 5, lines 7-18 teach that the skin layer and substrate layer are formed on the outer surface of the polystyrene layer, so that the inner surface of the polystyrene layer forms the interior surface of the culture chamber. The skin layer is formed from polyethylene copolymers and polypropylene copolymers. Column 4, lines 11-46 indicate that substrate layer is anywhere from 0-40% ethylene vinyl acetate copolymer. It is an intrinsic feature of the invention that the composition of the substrate and polystyrene layers can be manipulated in order to achieve any desired polymer distribution.

The claimed weight ratios are simply result effective variables. In the absence of new or unexpected results, it would have been obvious to optimize the composition of the substrate and skin layers. This optimization could simply be accomplished by producing different compositions and testing their ability to be used in cell culturing. See *In re Boesch*, 617 F.2d 272, 205 USPQ 215 (CCPA 1980).

With respect to claims 19-21, 23-26 and 51-53, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith states in column 2, lines 24-31 that the polystyrene layer (1st layer) has a thickness within the range of 0.0001 inches to 0.001 inches. Column 4, lines 47-56 indicate that the substrate layer (2nd layer) has a

thickness of 0.004 inches to 0.025 inches. Column 4, lines 11-46 teach that the second layer is a multi-component polymer blend that includes styrene and hydrocarbon copolymer. Figure 2 indicates that the gas permeable EVA material is used in the construction of both the first and second sidewalls. The nature of the invention regarding copolymer content and layer thickness has already been described.

With respect to claims 27-32, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith states in column 2, lines 39-51 that the culture container has a oxygen permeability of 9-15 Barrers, a carbon dioxide permeability of 40-80 Barrers, a nitrogen permeability of 10-100 Barrers and a water vapor transmission rate of less than 20 (g mil/100 in^2/day). Column 5, line 49 to column 6, line 8 indicates that the first and second sidewalls have a flexural modulus of 10,000-30,000 psi, and that the sidewalls are optically clear. The container is radiation sterilizable. Column 7, lines 39-44 indicate that at least one port (Figure 9:40) provides access to the containment area.

With respect to claims 34 and 35, Smith, Toner/Turner and Codner disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In addition, Smith states in column 6, line 66 to column 7, line 19 that the inside surfaces of the culture container can be modified in order to determine to what areas cells are allowed to adhere. Accordingly, it would have been obvious to apply the fibrin matrix disclosed by Toner to any part of the container surface that is desired to promote cell adhesion. This intrinsically could pertain to the entire inner surface of the container, or just specific regions of the inner surface. If the culture container is intended to

facilitate the growth of adherent cell types, then it would be obvious to apply the fibrin matrix to the entire sidewall interior surface. If the culture container is intended to facilitate the growth of adherent and non-adherent cell types, then it would be obvious to apply to fibrin matrix to just a part of the sidewall interior surface.

2) Claims 36-46 are rejected under 35 U.S.C. 103(a) as being unpatentable over Smith (US 5935847) in view of Toner (US 6759245)/Turner (US 5912177) and Codner (US 5686304) as applied to claim 1, and further in view of Delmotte (US 5989215).

With respect to claims 35-37, Smith, Toner/Turner and Codner disclose the invention set forth in the 35 U.S.C. 103 rejections above, however do not expressly disclose the nature of the fibrin matrix.

Delmotte discloses a method for forming a fibrin matrix that includes delivering a first solution of fibrinogen and factor XIII and a second solution of thrombin and calcium to a desired surface. This is disclosed in column 3, lines 31-44 and column 8, lines 3-15. In column 12, line 34 to column 13, line 20, Delmotte states that the amount of thrombin added to the fibrinogen solution is directly related to the pore size of the fibrin matrix product. Thrombin can be added in varying amounts in order to create a fibrin network characterized by pore diameters anywhere between 0.2-4 microns.

Smith, Toner/Turner, Codner and Delmotte are analogous art because they are from the same field of endeavor regarding cell culture systems.

At the time of the invention, it would have been obvious to form a fibrin matrix within the cell culture container disclosed by Smith and Toner by mixing a solution of fibrinogen with a

solution of thrombin. In column 4, line 57 to column 5, line 16, Delmotte states that by separating fibrinogen and thrombin into two separate solutions, one is able to more easily manipulate the concentrations of fibrinogen and thrombin to effect change in the characteristics of the resultant fibrin film. In this way, the concentration of thrombin can be readily changed in order to create a fibrin matrix with a desired pore size.

With respect to claims 38-46, Smith, Toner/Turner, Codner and Delmotte disclose the apparatus set forth in the 35 U.S.C. 103 rejection above. In column 7, lines 29-32, Delmotte teaches that the components of the fibrinogen and thrombin are derived from human plasma. It would have been obvious to utilize recombinant components of fibrinogen and thrombin, as well. When the fibrin matrix is used in a bioreactor and not for treating a human being, it is less important to use fibrinogen and thrombin attained from human blood plasma. Techniques for creating recombinant biomolecules are well known in the art.

With respect to claim 47, Smith, Toner/Turner, Codner and Delmotte disclose the apparatus set forth in claim 37 as set forth in the 35 U.S.C. 103 rejection above. In addition, Delmotte discloses in column 8, lines 3-29 that fibrin is made from a first solution containing 10-40 IU/ml of fibrinogen and factor XIII, and a second solution containing 3-10,000 IU/ml of thrombin and 45 micromoles/ml of calcium. Column 15, lines 1-15 disclose a method in which the fibrinogen and thrombin solutions are repeatedly applied to a surface in 0.3 ml increments. Column 18, lines 51-63 disclose a method in which 3.5 ml of the fibrinogen and thrombin

solutions are mixed to form a fibrin matrix. The fibrinogen and thrombin solutions are incubated, and the formed fibrin matrix has a pore size of anywhere between 0.2-4 microns.

Response to Arguments

Applicant's arguments filed 26 February 2008 with regard to the 35 U.S.C. 103 rejections involving the combination of Smith with either Toner or Turner and Codner have been fully considered but they are not persuasive.

Applicant's principle arguments are

(a) Smith is directed to a flexible, gas-permeable container, whereas Toner is directed to a rigid and impermeable cartridge. The suggested combination would change the principle of operation of the Smith apparatus.

In response to Applicant's arguments, please consider the following comments.

The previous Office Actions fully address this issue. The addition of a fibrin layer to the interior walls of Smith would not serve to substantially change the operation of the Smith culture bag.

(b) Smith does not disclose using ethylene vinyl acetate copolymer as any part of the inner cell growth surface layer. Moreover, Smith explicitly teaches away from an interior surface comprising an ethylene vinyl acetate copolymer by stating that the decay of the charge on EVA will render the container ineffective for growing adherent cells.

In response to Applicant's arguments, please consider the following comments.

It is agreed that Smith indicates that EVA is undesirable for the culture of adherent cells. However, Smith describes an embodiment in column 6, line 65 to column 7, line 20 and in Figure 7 indicating that at least one interior surface of the cell bag is constructed from materials compatible with non-adherent cells. EVA, as evidenced by Codner, is known in the art as a material capable of facilitating the growth of non-adherent cells. Accordingly, it would have been obvious to ensure that some areas of Smith's inner surface are covered by fibrin and polystyrene to encourage the growth of adherent cells, and it would have been obvious to ensure that other areas of Smith's inner surface are covered by ethylene vinyl acetate copolymers to promote the culture of non-adherent cells. The addition of fibrin and EVA to Smith's hybrid cell culture bag (Figure 7) would enhance the growth of both adherent and non-adherent cells.

(c) Codner fails to disclose the use of fibrin for growing cells anywhere in his disclosure.

In response to Applicant's arguments, please consider the following comments.

As noted above, Codner is not relied upon for teachings regarding the use of fibrin. The Toner and Turner references each describe that it is beneficial to provide a fibrin layer for the culture of adherent cells. Codner is merely relied upon as evidence that it is well established in the art to provide cell culture bags constructed from EVA materials.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Nathan A. Bowers whose telephone number is (571) 272-8613. The examiner can normally be reached on Monday-Friday 8 AM to 5 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gladys Corcoran can be reached on (571) 272-1214. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/William H. Beisner/
Primary Examiner, Art Unit 1797

/Nathan A Bowers/
Examiner, Art Unit 1797

EXHIBIT C

US005935847A

United States Patent [19]

Smith et al.

[11] Patent Number: 5,935,847

[45] Date of Patent: Aug. 10, 1999

[54] **MULTILAYER GAS-PERMEABLE CONTAINER FOR THE CULTURE OF ADHERENT AND NON-ADHERENT CELLS**

[75] Inventors: **Sidney T. Smith**, Lake Forest; **David V. Bacehowski**, Wildwood; **William Kolanko**, Grayslake; **Larry Rosenbaum**, Gurnee; **Stephen L. Smith**, Arlington Heights; **James G. Bender**, Lindenhurst; **Lecon Woo**, Libertyville; **Michael T. K. Ling**, Vernon Hills, all of Ill.

[73] Assignee: **Baxter International Inc.**, Deerfield, Ill.

[21] Appl. No.: **09/072,127**

[22] Filed: **May 4, 1998**

Related U.S. Application Data

[63] Continuation of application No. 08/549,632, Oct. 27, 1995, abandoned, which is a continuation-in-part of application No. 08/330,717, Oct. 28, 1994.

[51] **Int. Cl.**[6] **C12M 3/06**

[52] **U.S. Cl.** **435/297.5**; 435/304.1; 435/307.1; 604/408; 383/102; 383/109; 383/116; 428/35.2; 428/216; 428/220; 428/515; 428/516; 428/518

[58] **Field of Search** 435/297.5, 304.1–304.3, 435/307.1; 428/35.2, 35.7, 36.5, 220, 216, 332, 334–336, 337, 411.1, 474.4, 476.3, 500, 515, 517, 519; 383/102, 109, 113, 116; 604/408

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Primary Examiner—William H. Beisner

Attorney, Agent, or Firm—Mark J. Buonaiuto; Joseph A. Fuchs

[57] **ABSTRACT**

A multi-layer, flexible, gas-permeable film (**10**) suitable for forming a cell culture container (**20**), the film (**10**) comprising a first layer (**12**) composed of a polystyrene having a thickness within the range of **0.0001** inches to about 0.0010 inches and, a second layer (**14**) adhered to the first layer (**12**) composed of a polymer material having a thickness within the range of 0.004 inches to about 0.025 inches.

37 Claims, 4 Drawing Sheets

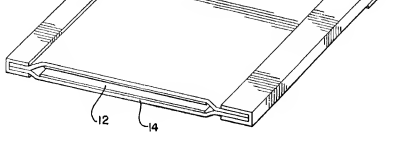

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FIG. 1

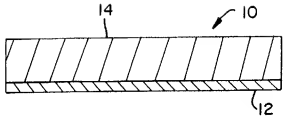


FIG. 2

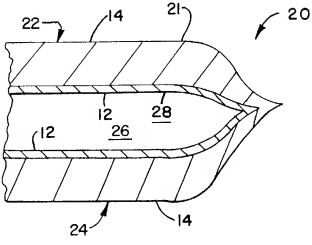


FIG. 3

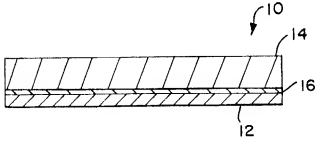


FIG. 4

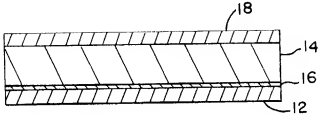


FIG. 5

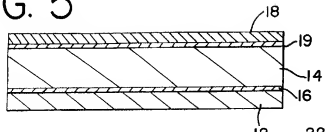


FIG. 6

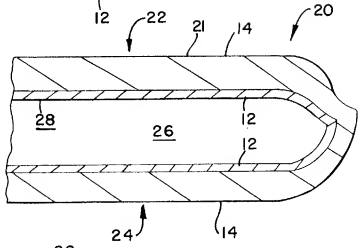


FIG. 7

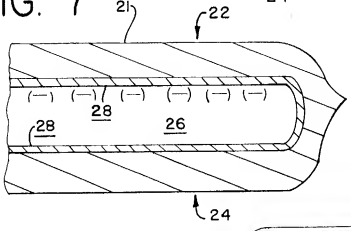


FIG. 7a

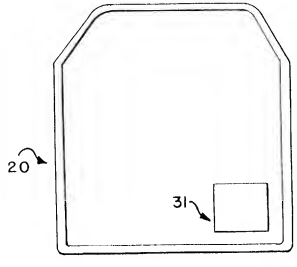


FIG. 8

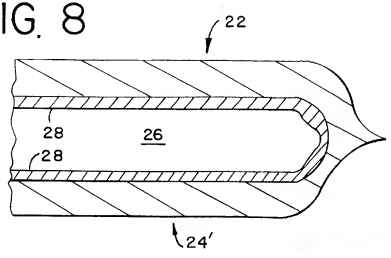


FIG. 9

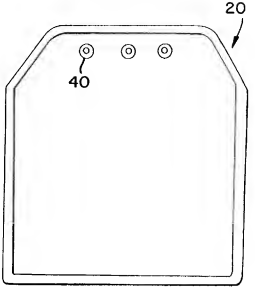


FIG. 10a

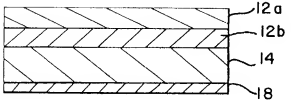


FIG. 10b

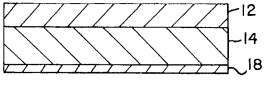


FIG.11a

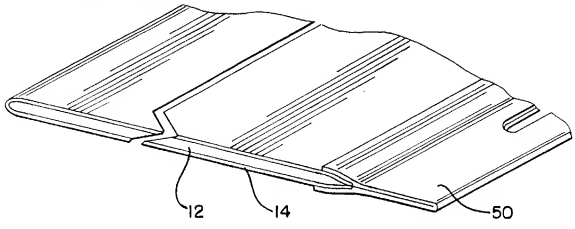
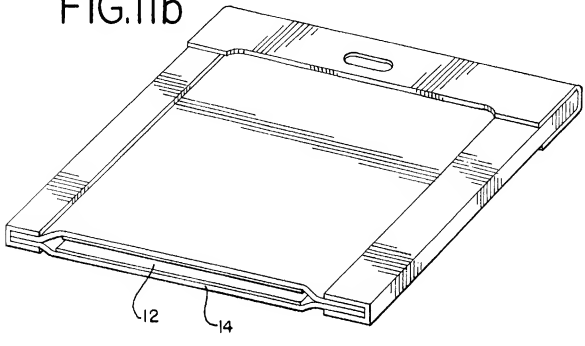


FIG.11b



MULTILAYER GAS-PERMEABLE CONTAINER FOR THE CULTURE OF ADHERENT AND NON-ADHERENT CELLS

RELATED APPLICATION

This application is a continuation of U.S. application Ser. No. 08/549,632 filed on Oct. 27, 1995, now abandoned, entitled "Multilayer Gas-Permeable Container For the Culture of Adherent and Non-Adherent Cells" which is a continuation-in-part from U.S. application Ser. No. 08/330,717, filed on Oct. 28, 1994, entitled "Multilayer Gas-Permeable Container For the Culture of Adherent and Non-Adherent Cells." The disclosure of U.S. application Ser. Nos. 08/330,770 and 08/549,632 are hereby incorporated by reference, and made a part hereof.

TECHNICAL FIELD

This invention relates to multi-layer films, and containers formed therefrom for the in vitro culture of cells. Specifically, the invention is directed to a multilayer, flexible, gas permeable container having an inner growing surface of polystyrene, which is conducive to the culture of cells.

BACKGROUND ART

There are two major types of cells grown in vitro; suspension cells (anchorage-independent cells); and adherent cells (anchorage-dependent cells). Suspension or anchorage-independent cells can multiply, in vitro, without being attached to a surface. In contrast, adherent cells require attachment to a surface in order to grow in vitro. Additionally, some non-adherent cells grow best on a surface that promotes adherent cell growth.

It is known to grow adherent cells, in vitro, in polystyrene flasks. Polystyrene is the most common type of plastic used in the manufacture of rigid, gas impermeable cell culture flasks or plates. It is thought that polystyrene promotes the growth of adherent cells because of its ability to maintain electrostatic charges on its surface which attract oppositely charged proteins on the cell surfaces. However, to date, the available polystyrene culture containers have been of the rigid flask or plate type because polystyrene is known in the art as a rigid, gas-impermeable plastic.

Cells are commonly cultured in a growth medium within polystyrene or other containers placed in enclosed incubators. In addition to providing a certain degree of isolation from pathogens, the incubators maintain a constant temperature, usually 37° C., and a constant gas mixture. The gas mixture must be optimized for a given cell type, and be controlled for at least two parameters: (1) partial pressure of oxygen (pO_2) to serve the aerobic needs of the cells, and (2) partial pressure of carbon dioxide (pCO_2) to maintain the pH of the growth medium. Since the known types of rigid cell culture containers are gas impermeable, their lids or caps are not sealed onto the containers. Rather, they are offset sufficiently to allow gas exchange through a gap or vent between the cap and the container. Such a container is disadvantageous for clinical uses because the vent might allow contamination of the culture or lead to accidents involving biohazardous agents.

In addition to polystyrene flasks, others have constructed flexible, breathable containers for containing adherent cells to be grown in vitro. For example, the commonly assigned U.S. Pat. No. 4,939,151 provides a gas-permeable bag with at least one access port. This allows for a closed system (ie., one without a vent). The bag disclosed in the '151 Patent is constructed from two side walls. The first side wall is made of ethylene-vinyl acetate ("EVA") which may be positively or negatively charged. The second side wall is constructed from a gas permeable film such as ethylene-vinyl acetate or a polyolefin. The first side wall is sealed to the second side wall along their edges. While EVA can hold an electrostatic charge, the charge has the undesirable tendency to decay over time. Eventually, the decay of the charge on EVA will render the container ineffective for growing adherent cells. Rigid styrene flasks with an electrostatic charge are known, and show less of a tendency to lose charge over time.

It has been found that the cell growth rate within a sealed container may be influenced by the gas permeability characteristics of the container walls. The optimal gas requirements, however, vary by cell type and over the culture period. Thus, it is desirable to be able to adjust the gas permeability of the container. The polystyrene flask, and the flexible flask which is entirely constructed from a monofilm, do not provide for such adjustability.

SUMMARY OF THE INVENTION AND OBJECTS

The present invention provides a multi-layer, co-extruded film suitable for producing gas-permeable cell culture bags. The film has an ultra-thin first layer of polystyrene having a thickness from about 0.0001 inches to about 0.0010 inches. The film has a second layer adhered to the first layer made of a polyolefin. The polyolefin acts as a flexible substrate for the polystyrene to provide a flexible, gas permeable film. Thus, the second layer is sometimes referred to as the substrate layer.

The film may also have an adhesive tie layer interposed between the first and second layers. The film may also have one or more additional outer layers of polyolefin (such as polypropylene or polyethylene) to provide strength and scratch resistance, as well as additional tie layers interposed between these additional layers.

The film most preferably has the following physical characteristics: (1) a mechanical modulus of between about 10,000 and 30,000 psi (ASTM D 790); (2) an oxygen permeability within the range of about 9–15 Barrers; (3) a carbon dioxide permeability of 40–80 Barrers; (4) a nitrogen permeability of 10–100 Barrers, and (5) a water vapor transmission rate of not more than 20 (g mil/100 in^2/day). Optionally, the film should have an optical clarity of between about 0.1% to about 10% as measured by a Hazometer in accordance with ASTM D1003. For adherent cell culture, preferably, the growth surface should have a positive or a negative greater than 40 dynes/cm. This charge will be referred to as surface energy.

The present invention also provides a flexible, gas-permeable cell culture container constructed from the above described films, with the polystyrene layer forming the inner surface of the container.

Another aspect of the present invention provides a flexible, gas-permeable cell culture container whose gas permeability may be adjusted to best match the requirements of the cell being cultured in the container. The multi-layer structure of the present film allows one to vary the material of the second layer or substrate layer and its thickness to achieve the desired or predetermined gas permeability requirements for cell growth. Preferably, the type and thickness of the substrate layer and the thickness of the polystyrene layer will be selected to optimize cell growth.

Another aspect of the invention provides for various embodiments of culture containers some of which are

advantageous for growing adherent cells, non-adherent cells, and both.

Another aspect of the invention is to provide a flexible, gas permeable cell culture container having a first side that is suitable for growing adherent cells, a second side for growing non-adherent cells, and indicia on the container for indicating the first side from the second side.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a cross-sectional view of a two-layer, gas-permeable, flexible film of the invention;

FIG. **2** is a cross-sectional view of the flexible, gas-permeable cell culture container of the invention;

FIG. **3** is a cross-sectional view of a three-layer, gas-permeable, flexible film of the invention;

FIG. **4** is a cross-sectional view of a four-layer, gas-permeable, flexible film of the invention;

FIG. **5** is a cross-sectional view of a five-layer, gas-permeable, flexible film of the invention;

FIG. **6** is a cross-sectional view of a flexible, gas permeable container for the growth of non-adherent cells;

FIG. **7** is a cross-sectional view of a flexible, gas permeable container for the growth of both adherent and non-adherent cells;

FIG. **7***a* is a plan view of the container of FIG. **7** showing geometric indicia to distinguish the adherent side from the non-adherent side;

FIG. **8** is a cross-sectional view of a flexible, gas permeable container for the growth of non-adherent cells having a clear panel for inspection of the contents;

FIG. **9** is a plan view of a flexible, gas permeable container of the present invention having access ports;

FIG. **10***a* is a cross-sectional view of a four layered film of the present invention having a crystalline polystyrene and a high impact polystyrene;

FIG. **10***b* is a cross-sectional view of a three layered film of the present invention having a skin layer;

FIG. **11***a* is a perspective view of a container having a skirt attaching the outer surface of one container panel to the outer surface of an opposite container panel;

FIG. **11***b* is a perspective view of a container formed from two opposed panels each having a polystyrene inner layer deposited on a central portion of substrate layers to leave a peripheral portion of the substrate layers uncoated with polystyrene to form a strong seam.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a multi-layer, gas-permeable flexible film, having a surface layer formed of polystyrene, and a cell culture container constructed therefrom, having an inner surface of polystyrene.

1. The Film Components

FIG. 1 shows a two-layer film **10**, of which the first layer **12** of the film forms an inner cell growth surface when fabricated into a cell culture container **20** (FIG. **2**). The film **10** has an effective thickness to allow cell culture growth. The first layer **12** is an ultra-thin layer of polystyrene, preferably having a thickness from about 0.0001 inches to about 0.0010 inches, more preferably 0.0002 inches to about 0.0006 inches, and most preferably about 0.0004 inches. The polystyrene material may be selected from, but not limited to, polystyrenes such as high impact polystyrenes ("HIPS") which are a general purpose polystyrene modified by polybutadiene rubber. Such a polystyrene is sold by Dow Chemical Company under the product designation Styron 47827, Natural. It is also possible to impact modify styrene using styrene-butadiene-styrene block copolymers which is known by the tradename K-Resin. Crystalline polystyrenes meeting USP Class 6 criteria may also be employed, as may multiple layers of crystalline styrene and impact modified polystyrene.

The second layer **14** is composed of a polymer material such as a polyolefin or a mixture of polymer materials. Preferably, the second layer **14** includes a polymer alloy comprising three components: styrene-ethylene-butadiene-styrene ("SEBS") block copolymer (40%–85% by weight), ethylene vinyl acetate (0–40% by weight), and polypropylene (10%–40% by weight) as described in the commonly assigned U.S. Pat. No. 4,140,162 which is incorporated herein by reference. Such a polymer alloy is identified by Baxter International Inc. under the product designation PL-732® and fabricated into flexible containers under the tradename Lifecell®. The present invention contemplates using a plurality of layers of the same material or different material to make up the substrate layer. It is also desirable to use, for the second layer **14**, other three and four component polymer alloys such as those disclosed in co-pending, and commonly assigned patent application Ser. No. 08/153,823, now U.S. Pat. No. 5,849,843, which is incorporated herein by reference. One such group of polymer alloys consists of a first component of a polypropylene which constitutes approximately 30–60% by weight of the polymer alloy. The second component is preferably an ultra low density polyethylene or polybutene-1 which constitute approximately 25–50% by weight of the polymer alloy. The third component of the polymer alloy is preferably a dimer fatty acid polyamide (which should be interpreted to include their hydrogenated derivatives as well), which constitutes approximately 5–40% by weight of the polymer alloy. The fourth component is a compatibilizing polymer that may be selected from various block copolymers of styrene with dienes or alpha olefins; the compatibilizing polymers may be modified with minor amounts of chemically active functionalities such as maleic anhydride. For example, the compatibilizing polymer may be an SEBS block copolymer. The fourth component should constitute between 5–40% by weight of the polymer alloy.

Preferably, the second layer **14**, (which may sometimes be referred to as the substrate layer **14**) has a thickness within a range of about 0.004 inches to about 0.025 inches, more preferably 0.005 inches to about 0.012 inches, and most preferably 0.006 inches to about 0.008 inches. It is also possible to use multiple layers of the same substrate material, such as PL-732®, to compose the substrate layer **14**. Even if multiple layers are used in the second layer **14**, the combined thicknesses of the substrate layer components should still fall within the above-specified thickness ranges.

In another embodiment of the invention (FIG. **3**), the film may include a first tie layer **16** interposed between the first and second layers **12** and **14**. Preferably the first tie layer is a gas permeable olefin and more preferably an ethylene polymer containing vinyl acetate within the range of 16%–30% by weight and most preferably ethylene vinyl acetate with 28% vinyl acetate. Other examples of these polymers include those sold by Quantum Chemicals under the tradename Bynel. It is also possible to use SEBS block copolymers as the first tie layer **16** such as those sold by Shell Chemical Company under the tradename Kraton.

The first tie layer **16** adheres the first layer **12** to the second layer **14**. The first tie layer **16** has a thickness preferably within a range from about 0.0002 inches to about 0.0012 inches, more preferably from about 0.0004 inches to about 0.0010 inches, and most preferably about 0.0005 inches.

In yet another embodiment of the invention (FIG. **4**), the film shown in FIG. 3 may also have a skin layer **18** adhered to the second layer **14** opposite the first layer **12**, to form an outer skin which adds strength and scratch resistance to the film **10**. The skin layer **18** is preferably formed from homo and copolymers of polypropylene, more preferably polypropylene polymers codified by rubber. Such polymers would include those sold by Mitsui under the trade name Admer™. The skin layer **18** preferably has a thickness within a range of from about 0.0001 inches to about 0.0020 inches, and more preferably 0.0005 inches. The skin layer could also be composed of a polyethylene.

FIG. **5** shows the film of FIG. **4** except with a second tie layer **19** interposed between the skin layer **18** and the substrate layer **14** to adhere the skin layer **18** to the substrate layer **14**. The second tie layer **19** may be composed of similar components as identified for the first tie layer **16** such as modified polyethylenes.

FIG. **10***a* shows yet another film embodiment having four layers. The layers include a layer of crystalline polystyrene **12***a*, a layer of HIPS **12***b*, an ethylene vinyl acetate (having a vinyl acetate content between 16%–30%) substrate layer **14**, and a polyethylene skin layer **18**. The crystalline polystyrene **12***a* readily accepts and holds a surface energy. The thicknesses of each of the four layers should be consistent with that set forth above with the exception that the combined thickness of the crystalline polystyrene and HIPS should be within the range of thicknesses set forth for the first layer **12**. The layers **12***a* and **12***b* may be of the same or different thicknesses to form respectively symmetrical or asymmetrical stacks.

FIG. **10***b* shows the film of FIG. **10***a* but without the crystalline polystyrene layer. Thus, the film of FIG. **10***b* has a first layer **12** of HIPS, a substrate layer **14** of EVA and a skin layer **18** of polyethylene. A two layered film such as the one shown in FIG. 1 is also contemplated by the present invention having a substrate layer **14** of EVA and a first layer **12** of HIPS.

II. Construction of the Film and its Physical Characteristics

In forming the film **10** of FIG. **1**, the ultra-thin layer of polystyrene **12** is co-extruded on the substrate layer **14** using a typical feedblock co-extrusion method.

The resultant film **10** should have a flexural modulus preferably within the range of 5,000–300,000 psi, more preferably within the range of 10,000–200,000 psi, and most preferably 10,000–30,000 psi as measured in accordance with ASTM D 790. The film should have an oxygen permeability within the range of 7–30 Barrers, more preferably 8–20 Barrers, and most preferably 9–15 Barrers. A Barrer has units of (volume of gas in cm^3) (film thickness in cm)(1×10^{-10})/(time in seconds) (surface area of film in cm^2) (partial pressure of gas in cm of Hg). The film should have a carbon dioxide permeability within the range of 40–80 Barrers. The film should have a nitrogen permeability of 10–100 Barrers. The film **10** should have a water vapor transmission rate of not more than 20 (g mil/100 in^2/day). Preferably the film should have an optical clarity within the range of about 0.1%–10% as measured by a Hazometer in accordance with ASTM D1003. For adherent cell culture, preferably, the growth surface should have a surface energy of greater than 40 dynes/cm. Most adherent cells require a negatively charged surface; however, some adherent cells require a positively charged surface. The containers must also be capable of withstanding radiation sterilization at radiation levels commonly used in the industry for sterilization.

III. Fabrication of Flexible, Gas Permeable Cell Culture Containers

We turn now to the gas-permeable, flexible cell culture container (**20**, FIG. **2**) formed from the multilayer films described above. The cell culture container **20** includes a body **21** that is constructed from a first side wall **22** and a second side wall **24**. The side walls **22** and **24** are sealed along their edges to define a containment area **26** for containing the cell culture media and cells. The side walls **22** and **24** may be sealed by any conventional means such as using heated die and platen which may be followed by a chill die and platen as is well known in the industry. Also, the side walls **22** and **24** may be sealed using inductive welding which also is known in the industry. For containers constructed from films having as the substrate layer **14** the polymer alloy including the dimer fatty acid polyamide, radio frequency techniques may be used. However, the present invention should not be construed to be limited to using any one of these fabrication techniques unless otherwise specified in the claims.

It is possible to construct various flexible, gas permeable containers from the above film in conjunction with other materials.

It has been found that particularly strong seals may be achieved by using the sealing methods disclosed in the co-pending and commonly assigned patent application Ser. No. 08/330,717, which is incorporated herein by reference.

In particular, as shown in FIGS. **11***a* and **11***b*,substrate layers of two films are attached to one another without attaching the polystyrene layers **12** to one another. As is shown in FIG. **11***a*, a skirt **50**, composed of the same material as the substrate layer **14**, attaches the respective outer surfaces of each of the layers **14** to one another. FIG. **11***b* shows a container constructed from a film having components as described above except the polystyrene layer **12** is deposited on a central portion of the substrate layer **14** leaving a marginal portion of the substrate layer **14** uncoated by the polystyrene. The container is formed by attaching the marginal edges together to form a seam of substrate material bonded to substrate material.

Cell culture containers **20** fabricated using these preferred methods have been found to be sufficiently strong to withstand centrifuging even over an extended period of time at high gravitational forces.

A. The Non-Adherent Cell Culture Container

FIG. **6** shows a flexible, gas permeable cell culture container **10** especially useful for the growth of non-adherent cells. The container **10** is constructed from folding and sealing the substrate layers **14** of the film shown in FIG. **1** to define a containment area **30**. The first layer of polystyrene **12** faces the containment area **26**.

B. The Hybrid Cell Culture Bag

FIG. **7** shows a flexible, gas permeable cell culture container **10** which is suitable for the growth of both

adherent and non-adherent cell types. This container is essentially the same as the container set forth in FIG. **6** except the first side wall **22** inner surface **28** is charged with a surface energy of greater than 40 dynes/cm and preferably about 60 dynes/cm. The charge shown is negative; however, it could have a positive charge depending upon the type of cell to be cultured. The first side wall **22** with the charge is suitable for growing adherent cells and the second side wall **24** is suitable for growing non-adherent cells. It is desirable to use some indicia **31** to indicate the charged side from the uncharged side such as the perimeter geometry of the cell culture container (See FIG. **7***a*). This would include such structural features as a rounding of corners or notching of any portion of the container, or in any way varying the shape or structural features of the container to indicate the charged side from the uncharged side. It is also possible to have a raised or embossed area on one side of the container. It is also possible to use color coding or other printed indicia for distinguishing the charged and uncharged sides **22** and **24**.

C. Non-Adherent Cell Culture Container with a Clear Panel

FIG. **8** shows a flexible, gas permeable cell culture container having a first side wall **22** constructed from the film shown in FIG. **1**. The second side wall **24'** is constructed from a film having a substrate **30** of ethylene vinyl acetate having a vinyl acetate content of 18%±2% and an inner layer **28'** of HIPS. The first and second side walls **22** and **24'** are bonded along their edges as set forth above or by any suitable method. The second side wall **24'** has an optical clarity as measured by a Hazometer in accordance with ASTM D1003 within the range of 0.1%–10% which provides for ease of viewing the cells using a microscope or with the naked eye. The first side wall **22** would serve as the cell growth surface for non-adherent cells. It is also possible to apply a surface energy to the inner surface **28** of the side wall **22** to provide for a growing surface for adherent cells.

Preferably each of the containers **20**, shown in FIGS. **6–8**, will include access ports **40** as shown in FIG. **9**. The access ports **40** facilitate filling and emptying of the container **20** of cells or cell culture media without interrupting the closed system. Of course, any number of access ports can be provided as well as a tube set assembly, or the like.

D. Other Containers

It is also desirable to construct containers using the films shown in FIGS. **3–5** and **10***a–10b*.

IV. Method of Providing Adjustable Gas Permeability

It is desirable to construct a flexible cell culture container **10** having a predetermined gas permeability. The predetermined gas permeability selected promotes cell growth within the container **10**. Preferably, the selected permeability optimizes cell growth.

The gas permeability depends upon the types of polymers substrate layer, the thickness of the individual layers, and the overall thickness of the film.

Thus, the method of constructing or fabricating a gas permeable, cell growth container having a predetermined gas permeability comprises the following steps: providing a polystyrene, providing an appropriate polymer substrate layer, and coextruding the polystyrene and the polymer producing a layered film having a gas permeability to effect cell growth. Preferably, the cell growth will be optimal.

EXAMPLE 1

Films in accordance with the present invention were coextruded from a 0.0003 inch thick layer of polystyrene (K-Resin or HIPS) on a 0.0075 inch thick layer of polyolefin alloy (PL-732® or PL-269®). A portion of the 732®/HIPS film was corona discharge treated (good results have also been achieved without corona discharge as shown in Table 1 below). The film was formed into a flexible container or bag using a heat seal process. A length of film was cut from a roll of coextruded film. Port fitments, described in the commonly assigned U.S. Pat. No. 4,327,726, which is incorporated herein by reference, were heat sealed to the film near the midpoint of the length. The film was folded across the width of the sheet, near the sealed port fitments. The folded sheet with port fitments was placed on a heated brass platen and heat sealed using a heated brass die. The die and platen were operated at a constant temperature, the die at 280° F., and the platen at 370° F. No chilling dies or devices were employed. After sealing the container, the container was removed from the platen and allowed to air cool. Port closures were solvent bonded to the port fitments as is known in the art.

The bags were radiation sterilized and employed in cell culture studies of human progenitor cells. The bags were used to culture progenitor cells in a 10–12 day period in vitro culture. Progenitor cells were derived from purified CD34+ cells (stem cells) collected from peripheral blood as mobilized stem cells during a leukopheresis procedure. The cells were cultured via a process described in co-pending and commonly assigned patent application Ser. No. 07/855,295, now abandoned, which is incorporated herein by reference. Coextruded cell culture bags were seeded at a cell density of $0.1–1\times10^5$ (cells/ml). Coextruded culture bags were able to support an increase in the total number of viable cells during the 10–12 day culture period. Typical increases of 40–70 fold were observed. Cell proliferation in excess of 100 fold has been obtained in the culture bags using recombinant growth factors known to support progenitor cell growth in vitro. In addition to the increase in cell number, the culture bags were able to support an increase in Colony Forming Cells ("CFC") and early granulocytes as determined by flow cytometry and cytological staining. Early granulocytes are considered nonadherent cells that grow well in an adherent cell environment.

EXAMPLE 2

A film in accordance with the present invention was coextruded with a 0.0003–0.0005 inch thick layer of high impact modified polystyrene on a 0.0075–0.0080 inch thick layer of polyolefin alloy (PL-732®). The film was formed into a flexible container or bag using a heat seal process. The flexible container was inserted into an envelope composed of PL-732® of between 0.010–0.011 inches thick, and the envelope was heat sealed to the container around the periphery.

The composite container was used to culture progenitor cells over a three day period in vitro culture. Progenitor cells were derived from purified CD34+ cells (stem cells) collected from peripheral blood as mobilized stem cells during a leukopheresis procedure. The cells were cultured via a process described in co-pending and commonly assigned patent application Ser. No. 07/855,295, now abandoned, which is incorporated herein by reference. Coextruded composite containers were seeded at a cell density of 1×10^5 (cells/ml). After three days, the culture density was 19.5×10^5 (cell/ml) with 82% of the CD 34+ cells viable.

These growth data compare favorably to a container made in accordance with Example 1 which, in the same experiment, had a culture density of 17.8×10^5 (cell/ml) at the end of the three day culture period.

EXAMPLE 3

Films made in accordance with the present invention were coextruded and were used to culture progenitor cells over a ten day period in vitro culture (X-Vivo 10+10 mg/ml HSA with IL3, G-CSF, GM-CSF at 1000 U/ml each and SCF at 20 ng/ml). Progenitor cells were derived from purified CD34+ cells (stem cells) collected from peripheral blood as mobilized stem cells during a leukopheresis proeedure. The cell proliferation data for various containers are set forth in the Tables 1 and 2 for two separate experiments.

TABLE 1

Bag Type and Treatment	Total Cells	P.I.*	% Viable	Viable Cells
PL-269** + K-Resin	6.3 E + 06	21	75.85	4.8 E + 06
PL-732 + K-Resin	2.1 E + 07	71	85.87	1.8 E + 07
PL-732 + HIPS	3.3 E + 07	111	87.08	2.9 E + 07
PL-732 + HIPS/CD***	3.1 E + 07	102	82.89	2.5 E + 07
TEFLON ®	3.4 E + 07	114	86.16	2.9 E + 07
Polystyrene Control	4.0 E + 07	132	82.24	3.3 E + 07

TABLE 2

Bag Type and Treatment	Total Cells	P.I.*	% Viable	Viable Cells
PL-269** + K-Resin	1.2 E + 07	20	94.26	1.1 E + 07
PL-732 + K-Resin	1.5 E + 07	26	95.39	1.5 E + 07
PL-732 + HIPS	2.1 E + 07	35	96.01	2.0 E + 07
PL-732 + HIPS/CD***	2.4 E + 07	40	95.88	2.3 E + 07
TEFLON ®	2.3 E + 07	39	94.68	2.2 E + 07
Polystyrene Contiol	2.7 E + 07	46	94.39	2.6 E + 07

*P.I. stands for Proliferation Index which indicates the fold increase in the cell growth. Thus, a proliferation index of 2 indicates a doubling in the number of cells.
**PL 269 is EVA (vinyl acetate 18%).
***CD stands for corona discharge treated.

EXAMPLE 4

Containers were coextruded from PL-732® and HIPS as set forth in Example 1. PL-732® containers and Teflon® containers are commercially available. These containers were used to culture lymphokine activated killer cells over a seven day period in AIM-V (serum free culture media) with IL-2 1000 U/ml growth factor. The results of this experiment are set forth in Table 3 wherein P.I.±S.D. stands for proliferation index plus or minus an indicated standard deviation.

TABLE 3

Bag Type and Treatment	P.I. ± S.D.
PL-732 ® + HIPS	3.0 ± 1.7
PL-732 ®	1.6 ± 1.2
TEFLON ®	3.6 ± 2.6

EXAMPLE 5

Containers were coextruded from PL-732® and HIPS as set forth in Example 1. PL-732® containers and Teflon® containers are commercially available. These containers were used to culture tumor infiltrating lymphocytes over a seven day period in AIM-V (serum free culture media) with IL-2 1000 U/ml growth factor. The results of this experiment are set forth in Table 4 wherein P.I.±S.D. stands for proliferation index plus or minus an indicated standard deviation.

TABLE 4

Bag Treatment	P.I. ± S.D.
PL-732 ®+ HIPS	9.0 ± 2.7
PL-732 ®	5.5 ± 5.7
TEFLON ®	2.8 ± 2.0

It is understood that, given the above description of the embodiments of the invention, various modifications may be made by one skilled in the art. Such modifications are intended to be encompassed by the claims below.

What is claimed is:

1. A multi-layer, flexible, gas-permeable film suitable for forming a cell culture container, the film comprising:

a first layer composed of a polystyrene having a thickness within the range of 0.0001 inches to about 0.0010 inches, the first layer defining an inner cell growth surface;

a second layer adhered to the first layer composed of a polymer alloy blend having multiple components, wherein at least one of the components is selected from the group consisting of styrene and diene copolymers and styrene and alpha-olefin copolymers, the second layer having a thickness within the range of 0.004 inches to about 0.025 inches and wherein the film has a modulus of elasticity of less than 30,000 psi.

2. The film of claim **1** wherein the polystyrene of the first layer is modified by polybutadiene rubber.

3. The film of claim **2** further including a third layer of crystalline polystyrene attached to the first layer on a side opposite the second layer.

4. The film of claim **1** wherein the first layer has a thickness in the range of about 0.0002 inches to about 0.0008 inches.

5. The film of claim **1** wherein the first layer has a thickness of about 0.0004 inches.

6. The film of claim **1** wherein the polymer alloy blend has three components, a first component is a styrene-ethylene-butadiene-styrene block copolymer, a second component is ethylene vinyl acetate, and a third component is polypropylene.

7. The film of claim **6** wherein the styrene-ethylene-butadiene-styrene block copolymer constitutes 40 to 85% by weight of the polymer alloy, the ethylene vinyl acetate constitutes 0 to 40% by weight of the polymer alloy, and the polypropylene constitutes 10 to 40% by weight of the polymer alloy.

8. The film of claim **1** wherein the second layer is composed of a four component polymer alloy blend.

9. The film of claim **8** wherein the four component polymer alloy has a first component of a polypropylene, a second component selected from the group consisting essentially of an ultra low density polycthylene and polybutene-1, a third component of a dimer fatty acid polyamide, and a fourth component of a styrene-ethylene-butadiene-styrene block copolymer.

10. The film of claim **9** wherein the first component constitutes within the range of 30–60% by weight of the polymer alloy, the second component constitutes within the range of 25%–50% by weight of the polymer alloy, the third

component constitutes within the range of 5%–40% by weight of the polymer alloy, and the fourth component constitutes 5%–40% by weight of the polymer alloy.

11. The film of claim **1** wherein the polymer alloy blend of the second layer includes an ethylene vinyl acetate.

12. The film of claim **11** further comprising a layer of polyethylene on the second layer on a side opposite the first layer.

13. The film of claim **1** further comprising a skin layer attached to the second layer on a side opposite the first layer.

14. The film of claim **13** wherein the skin layer is selected from the group of polypropylene and polyethylene.

15. The film of claim **14** wherein the skin layer is polyethylene.

16. The film of claim **14** wherein the skin layer is polypropylene.

17. The film of claim **14** wherein the skin layer has a thickness within the range of 0.0001 inches to about 0.002 inches.

18. The film of claim **17** wherein the skin layer has a thickness of about 0.0005 inches.

19. The film of claim **1** further comprising a tie layer interposed between the first and second layers, the tie layer providing adhesive compatibility between the first and second layers.

20. The film of claim **19** wherein the tie layer is composed of a gas permeable olefin.

21. The film of claim **20** wherein the gas permeable olefin is an ethylene polymer containing vinyl acetate within the range of 16%–32% by weight.

22. The film of claim **21** wherein the ethylene vinyl acetate has a vinyl acetate content of 28% by weight.

23. The film of claim **1** wherein the first layer has a surface energy greater than 40 dynes/cm.

24. A multi-layer, flexible, gas-permeable film suitable for forming a cell culture container, the film comprising:

a first layer composed of a polystyrene having a thickness within the range of 0.0001 inches to about 0.0010 inches, the first layer defining a cell growth surface;

a second layer adhered to the first layer composed of a polymer alloy blend material having multiple components, wherein at least only of the components is selected from the group consisting of styrene and diene copolymers and styrene and alpha-olefin copolymers; and,

wherein the film having physical properties within the range:

a>10,000 but<30,000

b>9 but<15

c>40 but<80

d>10 but<100

e<20

wherein:

a is the flexural modulus in psi of the film measured according to ASTM D-790;

b is the oxygen permeability in Barrers;

c is the earbon dioxide permeability in Barrers;

d is the nitrogen permeability in Barrers; and

e is the water vapor transmission rate in (g mil/100 in^2/day).

25. The film of claim **24** wherein the film has an optical clarity within the range of 0.1%–10% as measured by a Hazometer under ASTM D 1003.

26. The film of claim **24** wherein the second layer is composed of a polymer alloy blend material having a thickness within the range of 0.004 inches to about 0.025 inches.

27. The film of claim **24** wherein the first layer has a surface energy greater than 40 dynes/cm.

28. A method for fabricating a multi-layered film suitable for forming a container for culturing cells comprising the steps of:

providing a polystyrene to define a cell growth surface;

providing a polymer alloy blend material having multiple components, wherein at least one of the components is selected from the group consisting of styrene and diene copolymers and styrene and alpha-olefin copolymers; and,

coextruding the polystyrene and the polymer alloy blend material producing a layered film having a gas permeability to promote cell growth.

29. A method of claim **28** wherein the polystyrene layer has a thickness within the range of about 0.0001 to about 0.0010 inches, and wherein the polymer alloy blend material layer has a thickness within the range of about 0.004 to about 0.025 inches.

30. A flexible, gas-permeable cell culture container suitable for culturing cells, the container comprising:

a first side wall of the container begin suitable for growing adherent cells, the first side wall comprising a first layer of polystyrene having a thickness within the range of 0.0001 inches to about 0.0010 inches, and a second layer adhered to the first layer of a polymer alloy blend having multiple components, wherein at least one of the components is selected from the group consisting of styrene and diene copolymers and styrene and alpha-olefin copolymers, the second layer having a thickness within the range of 0.004 inches to about 0.025 inches;

a second side wall attached to the first side wall for growing non-adherent cells; and,

means associated with the container for distinguishing the first side wall from the second side wall.

31. The container of claim **30** wherein the means for distinguishing the first side wall from the second side wall is the geometry of the container.

32. The container of claim **30** wherein the first layer of the first sidewall of the container has a surface energy greater than 40 dynes/em.

33. A flexible, gas-permeable cell culture container suitable for culturing cells, the container comprising:

a first and second side wall each having edges, the first and second side walls being sealed together at their respective side wall edges to provide a containment area, wherein at least the first side walls composed of a first layer of polystyrene having a thickness within the range of 0.0002 inches to 0.0010 inches, the first layer facing an interior of the container to define a cell growth surface, and, a second layer adhered to the first layer comprising a polymer alloy blend material blend having multiple components, wherein at least one of the components is selected from the group consisting of styrene and diene copolymers and styrene and alpha-olefin copolymers; the second layer having a thickness within the range of 0.004 inches to about 0.025 inches.

34. The cell culture container of claim **33** further comprising at least one access port for accessing the containment area.

35. The cell culture container of claim **33** wherein the first layer has a surface energy of greater than 40 dynes/cm.

36. A multi-layer, flexible, gas-permeable film suitable for forming a cell culture container, the film comprising:

a first layer composed of 100% by weight of polystyrene having a thickness within the range of 0.0001 inches to

about 0.0010 inches, the first layer defining an inner cell growth surface; and,

a second layer adhered to the first layer composed of a polymer alloy blend having multiple components, wherein said polymer alloy blend comprises no more than 40% by weight of a polyolefin, wherein at least one of the components is selected from the group consisting of styrene and diene copolymers and styrene and alpha-olefin copolymers, the second layer having a thickness within the range of 0.004 inches to about 0.025 inches.

37. A multi-layer, flexible, gas-permeable film suitable for forming a cell culture container, the film comprising:

a first layer composed of 100% by weight of polystyrene having a thickness within the range of 0.0001 inches to about 0.0010 inches, the first layer defining an inner cell growth surface; and,

a second layer adhered to the first layer composed of a polymer alloy blend having multiple components, wherein at least one of the components comprises at least 40% to 85% by weight of the polymer alloy blend and is selected from the group consisting of styrene and diene copolymers and styrene and alpha-olefin copolymers, the second layer having a thickness within the range of 0.004 inches to about 0.025 inches.

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